STUDIES ON WATER BUFFALO (*Bos indicus*) PLASMA ALPHA-2-MACROGLOBULIN

THESIS

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BY

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Dedicated

To

My Parents

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CERTIFICATE

I certify that the work presented in this thesis has been carried out by **Ms. Atia Sheereen** under my Supervision and is suitable for submission for the award of Ph.D. degree in Biochemistry.

(M. Saleemuddin)
Professor of Biochemistry

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CONTENTS

LIST OF ABBREVIATIONS

αMs	Alpha-macroglobulins
α_1M	Alpha-1-macroglobulin
α_2M	Alpha-2-macroglobulin
α_1I_3	Alpha-1-inhibitor-3
α_2MR	Alpha-2-macroglobulin receptor
BAPNA	Benzoyl-DL-arginine-p-nitroanilide
Cis-DDP	Cis-dichlorodiamine platinum II
DTNB	5,5'. Dithiobis (2-nitro benzoic acid)
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetraacetic acid
MUG	Murinoglobulin
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet derived growth factor
PMSF	Phenyl methyl sulphonyl fluoride
PZP	Pregnancy zone protein
SDS	Sodium dodecyl sulphate
SBTI	Soyabean trypsin inhibitor
NaSCN	Sodium thiocyanate
TCA	Trichloro acetic acid
TNF - α	Tumour necrosis factor α
TNF - β_1	Tumour necrosis factor β_1
TNF - β_2	Tumour necrosis factor β_2

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Introduction

1.0 INTRODUCTION

1.1 General

Proteinase binding α-macroglobulins (αMs) constitute a large group of glycoproteins that occur in the blood and other body fluids of nearly all the studied vertebrates, in the egg whites of reptiles and birds as well as in few higher invertebrates (Sottrup-Jensen, 1989). The αMs should ideally be considered sophisticated binding proteins activated by proteolysis. The αM super family that also includes complement C_3, C_4 and C_5 (Sottrup-Jensen, 1987; Dodds and Day, 1993) is characterized by the ability of its members to interfere with the action of all the four classes of endoproteinases thiol, serine, acid and metalloproteinases and usually by:

1. The presence of a particularly exposed stretch of amino acids termed bait region.
2. Ability to undergo conformational change on proteolysis at the bait region and bind the attacking proteinases.
3. Ability to shield the bound proteinase(s) from large molecular weight inhibitors/substrates, while retaining access towards those of smaller dimensions.
4. Occurrence of a protected internal β-cysteinyl-γ-glutamyl thiol ester linkage with a potential for covalent binding of the activating proteinases and nucleophiles.
5. Ability to expose an unexposed site on proteolysis that is recognised by the high affinity receptors present on several cell types.

αMs have attracted remarkable attention in view of their implication in a variety of biological functions including restricting proteolysis, functioning as back-up proteinase inhibitors, clearing of proteinases from circulation, modulating immune response and binding several hormones, growth factors, etc. Specific expression of some αMs under conditions like infection or pregnancy further reteriate their important role *in vivo*. Extensive investigations have been made on the structure, proteinase and nucleophile-induced alterations, behaviour under stress and disease of various αMs (Roberts, 1986; Sottrup-Jensen, 1989).

αMs have been identified in a large number of animal species and isolated, purified and characterised from several sources. While the amino acid sequence analysis suggest that proteins of the αM family are quite conserved, occurrence of interesting variations in molecular dimensions, quaternary structure, sensitivity towards proteinases and amines as well as in proteinase entrapment efficiencies in the inhibitor from a variety of animal sources suggest remarkable evolutionary adaptations. Work on human αMs has been reviewed extensively (Sottrup-Jensen, 1987; 1989; Chaudhari, 1993). Attempts have therefore been made to present a critical account of αMs primarily from animal species.

1.2 Structure of αMs

1.2.1 Molecular structure

The structure of human, several other vertebrate and few invertebrate αMs has been elucidated in remarkable details at all levels. Majority of the characterised αMs are tetrameric, comprising of identical subunits assembled as non-covalently associated dimers of two disulphide-bonded dimers. Reports of heterotetrameric, homodimeric, heterodimeric and monomeric αMs are also however available (Table 1.1).

It has been suggested that αMs, both proteinase inhibitors and complement components, have evolved from a common monomeric precursor (Sottrup-Jensen *et al.*, 1985). Starkey and Barrett (1982) hypothesized earlier that mammalian murinoglubulin (MUG) and non-mammalian monomeric αMs evolved directly from a common ancestral gene based on the general features of molecular evolution: the primitive gene emerges as a monomer, and then evolves into polymer with concomitant development of additional function. Surprisingly, monomeric α_2Ms have not been identified in non vertebrate and presumably amphibian represent the most primitive class in which they have been identified while their occurrence in rodents has been reported by several investigators. This suggest that the MUG is not a primitive form of tetrameric αM but rather has evolved under selective pressure which is different from that of the tetrameric paralogue

TABLE 1.1

SOME PROPERTIES OF α-MACROGLOBULINS FROM NON-HUMAN SOURCES

Phylum	Species	Type	Subunit Number	Molecular weight (KDa)	Proteinase binding / moles/mole αM	References
Mollusca	Octopus	α_2M	2	180	n.d.	Thogersen *et al.* (1992)
	Crayfish	α_2M	2	190	1	Hergenhahn *et al.* (1988)
Arthropoda	Horseshoe Crab	α_2M	2	185	1	Quigley and Armstrong (1983 a;b) Armstrong *et al.* (1991); Melchior *et al.*(1995); Armstrong *et al.*(1994).
	American Lobster	α_2M	2	180	1	Spycher *et al.* (1987)
Pisces	Plaice	α_2M	2	105 & 90	0.45	Starkey and Barrett (1982)
	Hag Fish	α_2M	2	180	n.d.	Osada *et al.* (1988)
	Rainbow Trout	α_2M	2	180	n.d.	Freedman (1991)
Amphibia	Salamander	α_1M	2	180	2	Sallenave and Bellot (1987)
	Frog	α_1M	4	180	< 1	Feldman and Pizzo (1985)
		α_2M	2	180	0.5	Feldman and Pizzo (1986)

		αM	1	180	n.d.	
Reptilia	Turtle	αM	2	180	n.d.	Osada *et al.* (1988)
		Ovomacroglobulin	2	180	n.d.	
	Cobra	αM	1	175	n.d.	Ikai *et al.* (1983)
	Crocodile	Ovomacroglobulin	2	180	n.d.	Ikai *et al.* (1985)
Aves	Ostrich	α_2M	4	195	1	Van-Jaarsveld *et al.* (1994)
	Duck	Ovomacroglobulin	4	180	1	Nagase *et al.* (1986)
	Turkey	αM	1	180	n.d.	Feldman and Pizzo (1984)
	Chicken	αM	1	180	1	Feldman and Pizzo (1984)
		Ovomacroglobulin	4	180	0.8	Kitamoto *et al.* (1982)
Mammalia	Hedgehog	α_2M	4	200	2	Picard (1966)
	Rabbit	α_1M	4	220	2	Lebreton de Vonne and Mouray (1968); Lebreton de Vonne *et al.* (1971)
		α_2M	4	220	1	
	Squirrel	α_2M	4	n.d.	n.d.	Dangott *et al.* (1983)
	Rat	α_1M	4	142 & 42	1.4	Scheufele and Koo (1982) Longberg Holm *et al.* (1987) Nelles and Schnebli (1982); Gautheir and Mouray (1976); Gordan (1976); Esnard *et al.* (1985); Longberg Holm *et al.*(1988)
		α_2M	4	190	1	
		α_1I_3	1	215	0.3-0.5	

						et al.(1988)
	Mouse	αM	4	163 & 35	0.98	Hudson *et al.* (1987); Hudson and Koo (1982)
		MUG	1	180	0.6	Van Leuven *et al.* (1982)
	Guinea Pig	α_2M	4	180	n.d.	Suzuki and Sinohara (1986)
		MUG	1	180	n.d.	Miyake *et al.* (1993).
	Hamster	MUG	1	180		Miyake *et al.* (1993)
	Dog	α_1M	4	n.d.	n.d.	Ohlsson (1971a;b)
		α_2M	4	n.d.	n.d.	Lavergne and Raynaud (1970)
	Horse	α_2M	4	180	2	Motoshima *et al.* (1988)
	Cow	α_2M	4	180	1	Dangott and Cunningham (1982) Nagasawa *et al.* (1970); Sujghara *et al.* (1971); Nakamura *et al.* (1977)
	Pig	α_1M	4	180	n.d.	Baumstark (1973); Tsuru *et al.* (1975); Jacquot – Armand and Guinand (1976)
		α_2M	4	180	2	
	Goat	α_2M	4	110 & 36	1	Khan *et al.* (1999).
	Mink	α_2M (Serum)	4			Skobel'tsyna *et al.* (1991) Rantamaki and Muller (1992)
		α_2M (Mastitis milk)	4			

(Iwasaki et al., 1996). They appear to be widespread among aves, reptiles, amphibia and mammals (Rubenstien et al., 1993; Miyake et al., 1993). Rat α_1I_3 is a structurally and functionally well characterized monomeric proteinase inhibitor, comprising of single polypeptide of 180 KDa. (Enghild *et al.*, 1989).

αMs appearing in the hemolymph of the invertebrate limulus belonging to the arachnid class is homodimeric (Spycher *et al.*, 1987), so are those of the other invertebrates such as horseshoe crab (Quigley and Armstrong, 1983a;b), octopus (Thogersen *et al.*, 1992), crayfish (Hergenhahn *et al.*, 1988; Stocker *et al.*, 1991). Homodimeric αMs have also been characterised from several mammalian and avian sources (Sottrup-Jensen, 1987) and include PZP of humans (Sand *et al.*, 1985; Christensen *et al.*, 1989), hag fish α_2M (Osada *et al.*, 1988), frog α_2M (Feldman and Pizzo, 1986) and crocodile ovostatin (Ikai *et al.*, 1985). Plaice αM is however heterodimeric comprising of disulphide linked 105KDa and 90 KDa peptides (Starkey and Barrett, 1982).

Starkey (1983) categorised the tetrameric αMs into type I and type II based on the nature of subunits. While type I αMs are composed of identical subunits, those with non identical subunits were grouped as type II. Human αMs, and those from rabbit (Lebreton de Vonne and Mouray, 1968), horse (Motoshima *et al.*, 1988), guinea pig (Suzuki and Sinohara, 1986a;b), cow (Nagawsa *et al.*, 1970), dog (Ohlsson, 1971a;b), chicken (Feldman and Pizzo, 1984a) and duck (Nagase *et al.*, 1986) are homotetrameric and hence of type I.

Mouse α_2M (Hudson, 1987) and rat α_1M (Schaeufele and Koo, 1982) are type II inhibitors, have basic architecture similar to human α_2M but the quarter subunits are made up of two unequal disulphide linked polypeptides chain. The mouse α_2M comprises of four heavy (163 KDa) and four light chains (35 KDa) while heavy and light chain in rat α_1M are of 140 KDa and 42 KDa (Hudson *et al.*, 1987; Longberg-Holm *et*

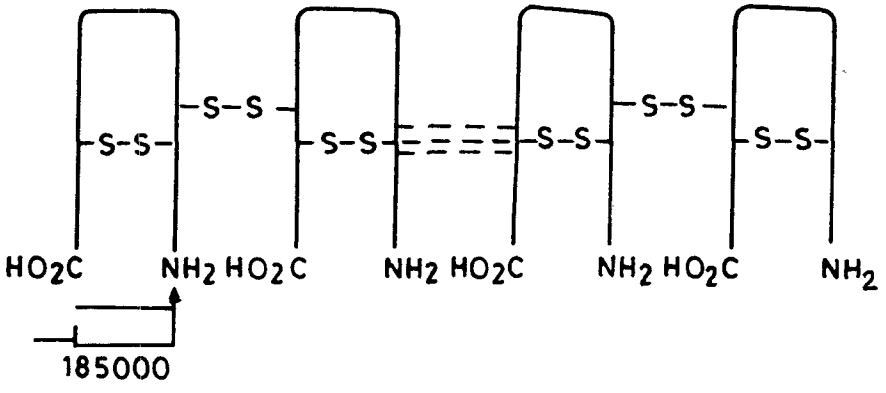
al., 1987). The proposed model for human and mouse α_2M representing type I and II αMs (Harpel, 1973; Steinbuch *et al.*, 1975; Barrett *et al.*, 1979; Hudson *et al.*, 1987) are shown in Fig.1.1.

Starkey and Barrett (1982) proposed that the single chain subunits αM homologues probably arose from ancestral protein having subunits of unequal sizes by one of the two mechanism. The first assumption that the structural genes for subunit I and II of αM are adjacent and that deletion of the stop sequence between these two genes resulted in their being transcribed as single m-RNA and is translated as single polypeptide corresponding to quarter subunit of α_2M. The second hypothesis suggests that αM homologue having larger and smaller subunit originally arose from single chain precursor by post translational cleavage by specific proteinase . A mutation in the amino acid sequence recognised by the specific proteinase could then give rise to a precursor protein that was resistant to proteolysis and persisted as 185 KDa subunit. Such post trannslational proteolytic processing has been proved in rat α_1M (Geiger et al., 1987).

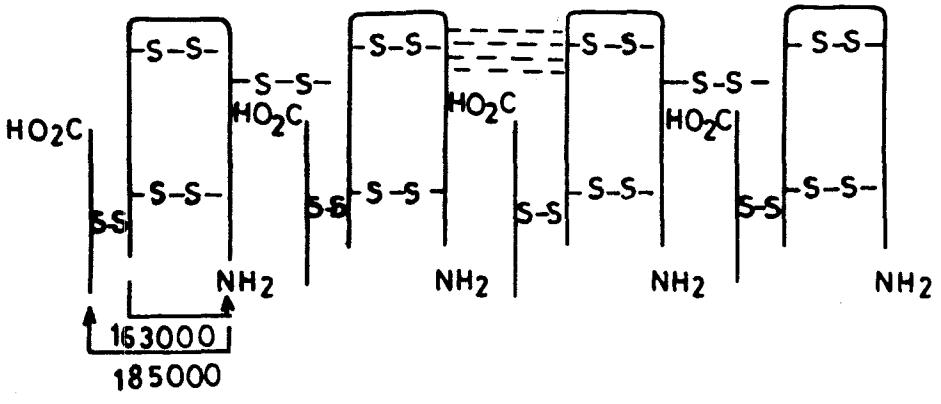
In the course of an experiment to isolate αM, murine plasma was shown to contain two different components having trypsin-protein esterase activity. The isolation and characterization of the two components revealed that one was previously known αM (Greene *et al.*, 1971; Hudson and Koo, 1982), whereas the other, tentatively named MUG, did not correspond to any of the known proteinase inhibitors in the mammalian plasmas. MUG clearly differed from mouse αM and human α_2M in antigenicity, molecular size and binding properties with trypsin, papain and thermolysin. (Saito and Sinohara, 1985 a).

MUG have been purified and characterised from the plasma of several other rodents such as guinea pig (Iwasakai *et al.*, 1996), rat (Saito and Sinohara, 1985 b) and hamster (Miyake *et al.*, 1993) and are characterised chemically and physiochemically. Rat plasma protein named α_1-inhibitor 3(α_1I_3) (Gauthier and Ohlsson, 1978) was thought

Figure 1.1 Proposed model for the structure of human (A) and mouse (B) α_2M.


S-S
S-S
S-S
S-S
S-S
S-S
S-S
HO2C
NH2
HO2C
NH2
HO2C
NH2
HO2C
NH2
185000

A


S-S
S-S
S-S
S-S
S-S
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S-S
S-S
S-S
S-S
S-S
S-S
HO2C
HO2C
HO2C
NH2
NH2
NH2
NH2
163000
185000

B

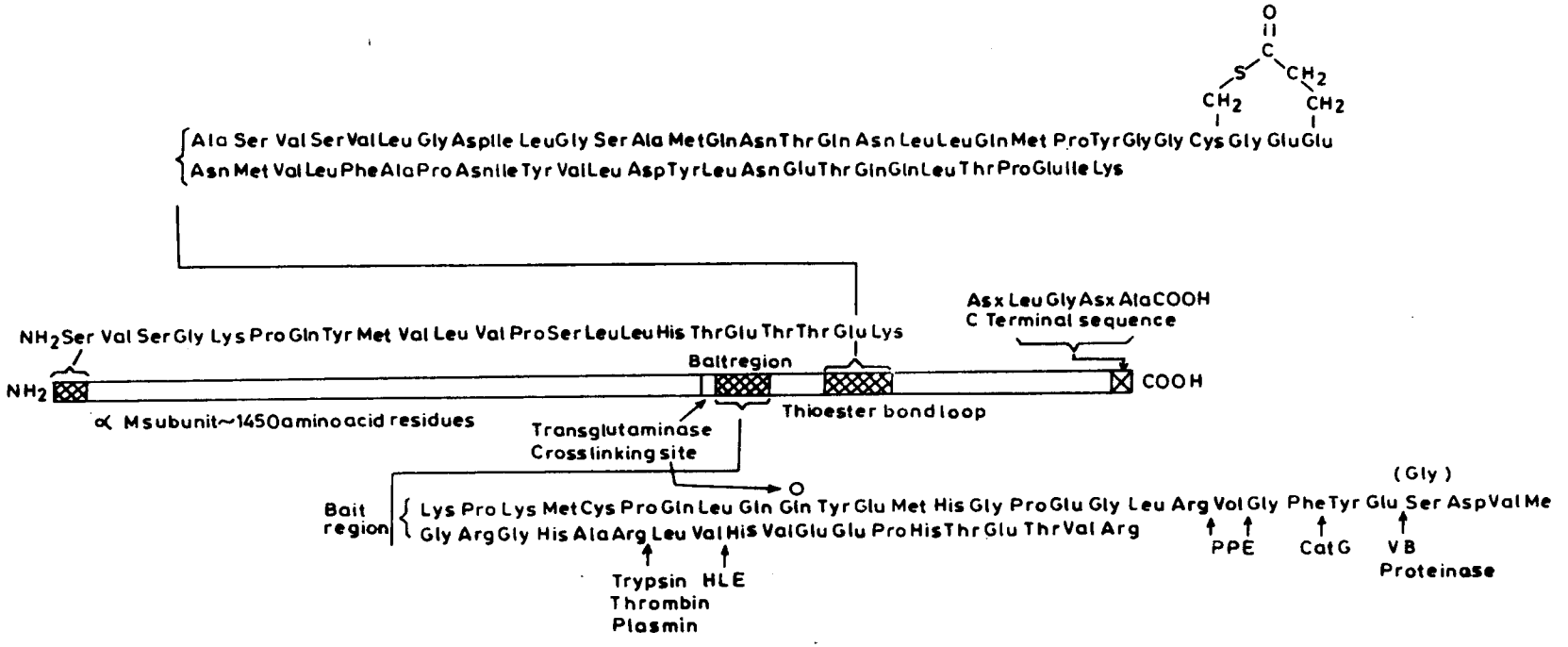
to be the rat counterpart of human inter-α-trypsin inhibitor, since they showed immunological cross reactivity (Esnard and Gauthier, 1980; Esnard *et al.*, 1985). However, it was later identified as a monomeric αM (Esnard *et al.*, 1985) that is a mixture of two isoforms, MUG I and MUG II (Saito and Sinohara, 1985a). MUGs have not however been detected so far in rabbit, dog, sheep, bovine, goat, pig or human (Saito and Sinohara, 1985a).

Complete amino acid sequence of the tetrameric human α_2M, rat α_2M, α_1M, dimeric human PZP and monomeric α_1I_3 are available (Sottrup-Jensen *et al.*, 1984; Kan *et al.*, 1985; Aiello *et al.*, 1988; Eggersten and Fey, 1989). A comparison of these sequences of the α_2Ms indicates that 41% of all residues are conserved which suggests that the three dimensional structures of αM subunits of the tetrameric, dimeric and monomeric forms may be similar. Lack of remarkable internal sequence similarities are also characteristic although a repeating pattern of predicted β-strands and turns throughout the sequence of human α_2M suggests a multiple domain structure largely composed of rather long β-barrel type domains. Sequences of the genes coding human and rat α_2M, and rat α_1I_3 are also available (Sottrup-Jensen, 1987 ; Eggersten *et al.*,1991; Kan *et al.*,1985).

1.2.2 The bait region

The bait region of αMs comprises of an exposed stretch of amino acids located approximately in the middle of the monomer that provides peptide bonds susceptible to cleavage by all classes of proteinases (Fig. 1.2). The αM bait region appears to be uniquely positioned in a manner that enables the adoption by the segment of a conformation that facilitates ready access to a wide variety of proteinases (Sottrup-Jensen, 1987; Sottrup-Jensen *et al.*, 1989). The bait region appears to be an irregular solvent exposed flexible structure (Sottrup-Jensen., 1989) and paramagnetic resonance of the human α_2M indicates that it is a relatively compact (Gettins *et al.*, 1988).

Figure 1.2 Schematic diagram of human α_2M subunit showing relative positions of the functionally important regions.


Ala Ser Val Ser Val Leu Gly Asp Ile Leu Gly Ser Ala Met Gln Asn Thr Gln Asn Leu Leu Gln Met Pro Tyr Gly Gly Cys Gly Glu Glu
Asn Met Val Leu Phe Ala Pro Asn Ile Tyr Val Leu Asp Tyr Leu Asn Glu Thr Gln Gln Leu Thr Pro Glu Ile Lys
NH2 Ser Val Ser Gly Lys Pro Gln Tyr Met Val Leu Val Pro Ser Leu Leu His Thr Glu Thr Thr Glu Lys
Asx Leu Gly Asx Ala COOH
C Terminal sequence
Bait region
NH2
COOH
α M subunit ~ 1450 amino acid residues
Thioester bond loop
Transglutaminase
Cross linking site
Bait region
Lys Pro Lys Met Cys Pro Gln Leu Gln Gln Tyr Glu Met His Gly Pro Glu Gly Leu Arg Val Gly Phe Tyr Glu Ser Asp Val Me
Gly Arg Gly His Ala Arg Leu Val His Val Glu Glu Pro His Thr Glu Thr Val Arg
(Gly)
PPE
CatG
VB
Proteinase
Trypsin
Thrombin
Plasmin
HLE

The bait region in human α_2M may be located very close (11-17^{0}A) to the thiol ester bond (Gettins *et al.* 1988) or positioned 20-25^{0}A away and may form a compact loop of two strands of β-sheets (Sottrup-Jensen *et al.*, 1990).

Remarkable variations between the length, composition and sequence of the bait regions of α_2M have been observed in various animal species, inspite of its apparent identical function. Thus, while the bait region of human α_2M is 39 amino acid long (Kan *et al.*, 1985; Sottrup-Jensen *et al.*, 1986), those of human PZP, rat α_1M, rat α_2M and α_1I_3 have respectively 49, 53, 52 and 32. residues (Sottrup-Jensen *et al.*, 1984; Sand *et al.*, 1985; Gehring *et al.*, 1987; Aiello *et al.*, 1988; Braciak *et al.*, 1988).

Over half the amino acid residues of the bait regions of αMs are hydrophobic (Kyte and Doolittle, 1982) but they are also generally rich in charged residues. The number of charged residues in the bait region is 9 in human α_2M, 6 in rat α_1M, 8 in rat α_2M, and 12 and 13 respectively in variants 1 and 2 of rat α_1I_3 (Sottrup-Jensen *et al.*, 1989). Human PZP with only three charged residues in the bait region appears to be an exception (Sottrup-Jensen *et al.*, 1984; Sand *et al.*, 1985). Glycine and proline residues appear generally abundant in the bait regions although those of rat α_2M and α_1I_3 have relatively lower content of the amino acids. (Sottrup-Jensen *et al.*, 1989). Considering the marked diversity in the amino acid composition/sequence it is not clear if the bait region of all αMs have similar conformations.

Inspite of the susceptibility of a wide variety of proteinases to inhibition and complex formation by the αMs, their rates of interaction with the inhibitor differ remarkably. For instance, while the rates of cleavage of human α_2M, rat α_1M and α_2M by the restrictive human fibroblast collagenase are high, those of rat α_1I_3 and human PZP are very low (Sottrup-Jensen, 1989). Rat α_1M shares 57.2 and 53 percent overall amino acid sequence identity with α_2M and α_1I_3 respectively yet they differ, remarkably in the sequence of the bait region (Eggertsen *et al.*, 1991). This suggests that *in vivo* each

inhibitor may actually address a specific of proteinases. Infact, the chicken ovostatin may be specific for metalloproteinases and the duck ovostatin for serine and metalloproteinases (Nagase *et al.*, 1986; Feldman and Pizzo, 1984a).

The bait region, apart from serving as a site for proteolysis for target proteinases also plays an important role in the non-covalent association of the half molecules into active tetrameric α_2M. A part of the C-terminal portion may be actually involved in the non covalent association of α_2M dimers into active tetramers (Gettins *et al.*, 1995). Perhaps relevant to the role of the bait region in interdimeric interactions is the observation that it is much harder to disrupt the non covalent interaction in the proteinase reacted α_2M, than in native α_2M. This suggest a major alteration in the interdimeric contacts on the proteolysis of the bait region (Pochon *et al.*, 1989; Pochon *et al.*, 1987). More recent findings by Gettins *et al.* (1995) implicate the bait region in the conformational changes that results in thiol ester activation and proteinase trapping.

Novel inhibitory specificities not exhibited by the native αMs have also been introduced in the inhibitor using recombinant DNA technology. Van Rompaey *et al.* (1995) obtained a recombinant of human α_2M (rα_2M) by introducing of a heptapeptide (Glu-Asn-Leu-Tyr-Phe-Gln-Gly)in the bait region. Unlike the unmodified inhibitor the rα_2M exhibited significant inhibitory action toward the tobacco etch virus (TEV) proteinase. In a more recent study Van Rampaey *et al.* (1997) created a human α_2M that was inhibitory towards a membrane bound proteinase which was recalcitrant to the action of the native α_2M.

1.2.3 The thiol ester

αMs and the complement proteins C_3 and C_4 are presumably the only known proteins that contain an internal thiol ester linkage (Sottrup-Jensen, 1987). The thiol ester appears to serve two important functions – to provide means of covalent binding to target proteinases and other proteins and mediate the large scale conformational rearrangements

that accounts for the engulfing of the challenging proteinases in the oligomeric form even in the absence of covalent binding (Gettins *et al.*, 1995).

In human α_2M, the thiol ester forms a 15 membered thiolactone ring involving the conserved segments Cys949-Gly-Glu-Glx952 (Tack *et al.*, 1980; Harrison *et al.*, 1981; Sottrup-Jensen *et al.*, 1985). The thiol ester is located in a small hydrophobic pocket shielded from the solvent and is accessible only to small molecular weight nucleophiles such as ammonium ion, methylamine and hydroxylamine (Gettins *et al.*, 1988; Larson *et al.*, 1987; Zhao *et al.*, 1988). Similar nucleophilic attack by the amino group at of the γ-carbonyl group of glutamic acid residue cleaves the peptide at this site at elevated temperature (Fig. 1.3.) (Barrett *et al.*, 1979; Harpel *et al.*, 1979; Howard, 1981). Bait region cleavage of monomeric and oligomeric αMs by proteinases however remarkably increases the reactivity of the thiol ester transforming it into a short lived nascent state in which it reacts to form a ε-lysyl-γ-glutamyl bond with a variety of nucleophiles including the side chain amino groups of the attacking proteinases (Fig. 1.4) or other proteins (Sottrup-Jensen, 1989; Borth and Luger, 1989; Ghetie *et al.*, 1991). In addition, several growth factors have been shown to be associated to αMs via the thiol ester linkage (Feige, 1996; Lysiak, 1995; Web, 1995). It has also been shown recently that covalent complexes formed between αMs and protein may facilitate antigen presentation by macrophages to the T-cells (Chu *et al.*,1994; Chu and Pizzo,1993). Formation of the covalent linkage however appears to be obligatory for the formation of inhibitor-proteinases complex only in case of the monomeric and possibly some dimeric forms (Saito and Sinohara, 1985a;b; Sottrup-Jensen, 1989; Longberg-Holm *et al.*, 1987).

Reaction of the thiol ester of human α_2M with the small nucleophiles results in a major conformational change in the molecule leading to the formation of a electrophoretically fast form which resembles that induced by the proteolytic attack on the bait region and accompanying cleavage of the thiol ester. Subtle difference between the fast forms resulting from methylamine treatment and bait region cleavage have however been observed (Delain *et al.*, 1988).

Figure 1.3 The structure of the thiolester bond loop in α_2M and reaction of amines with α_2M (upper reactions) and heat/alkali induced cleavage of the subunit chain (lower reactions).

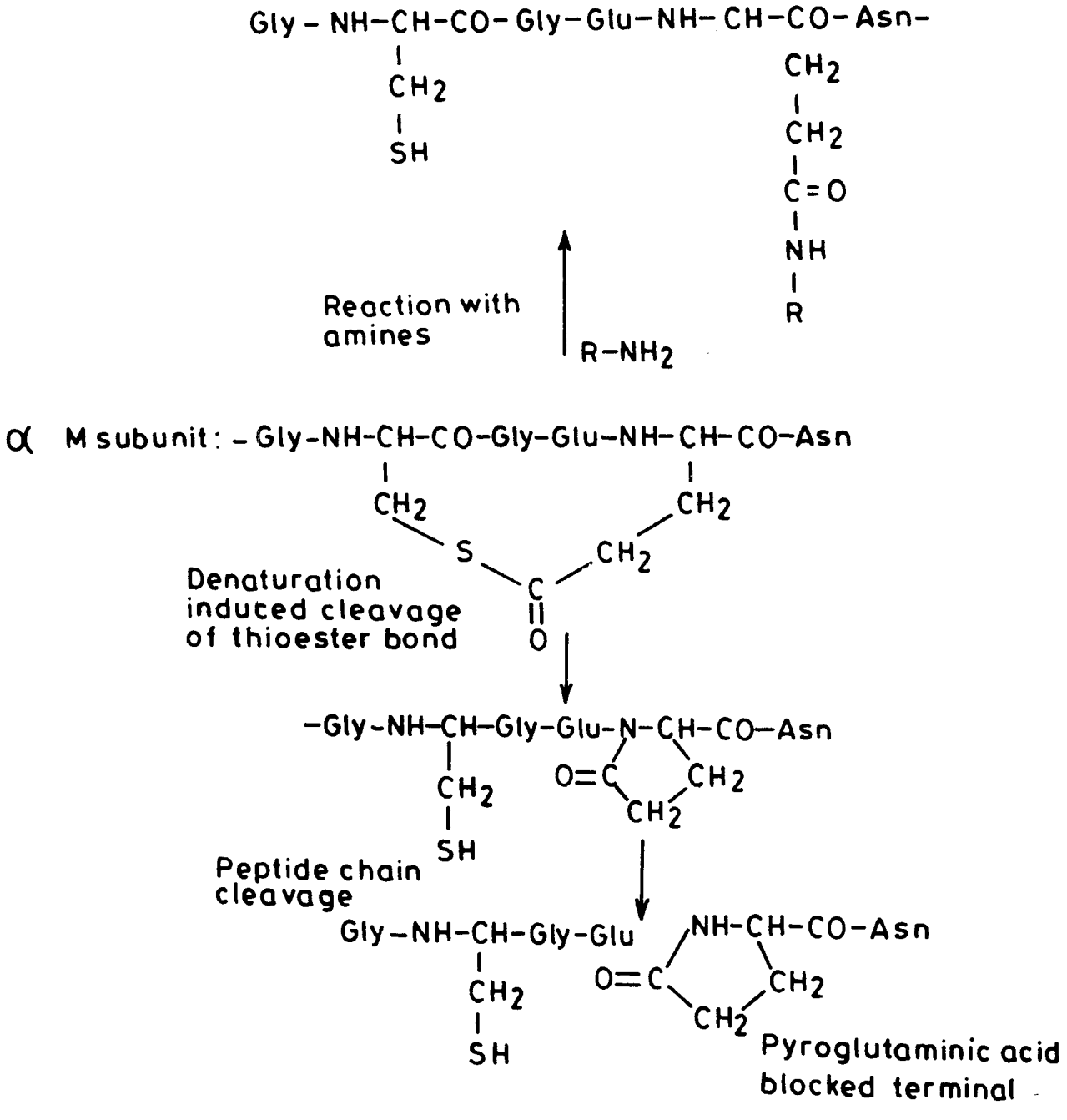
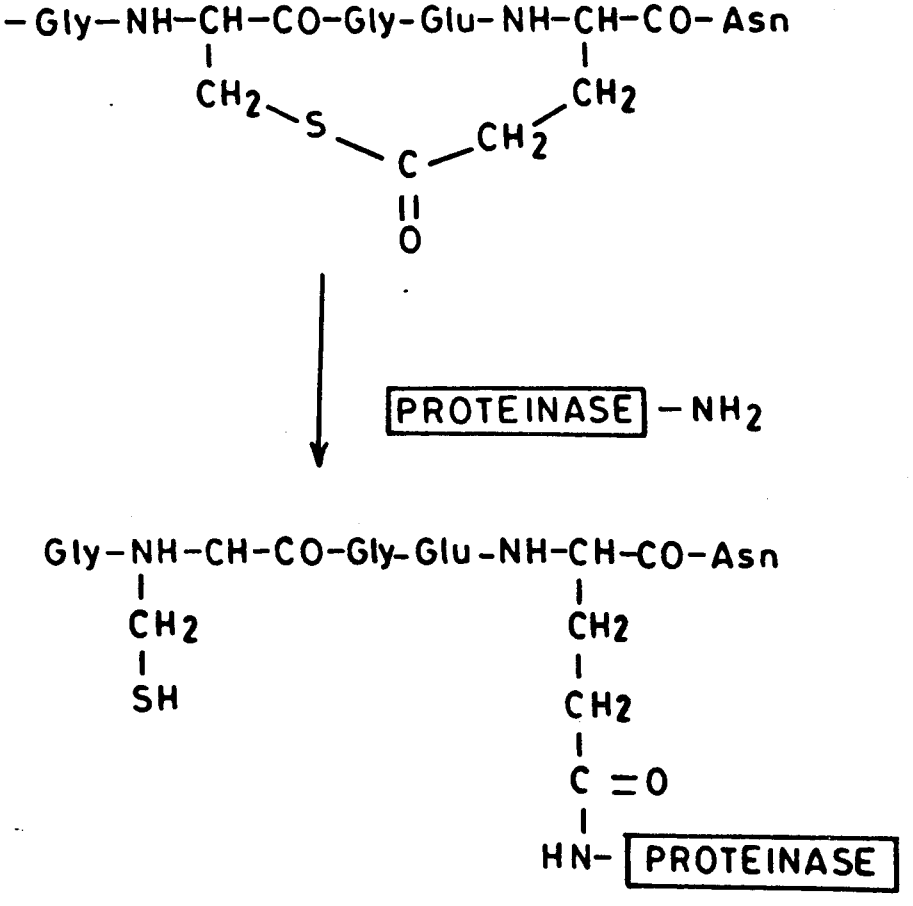

Gly - NH-CH-CO- Gly-Glu-NH- CH-CO- Asn-
CH2
SH
CH2
CH2
C=O
NH
R
Reaction with amines
R-NH2
α M subunit: - Gly-NH-CH-CO-Gly-Glu-NH-CH-CO-Asn
CH2
CH2
S
CH2
C
O
Denaturation induced cleavage of thioester bond
-Gly-NH-CH-Gly-Glu-N-CH-CO-Asn
CH2
SH
O=C
CH2
CH2
Peptide chain cleavage
Gly-NH-CH-Gly-Glu
NH-CH-CO-Asn
CH2
SH
O=C
CH2
CH2
Pyroglutaminic acid blocked terminal

Figure 1.4 Formation of covalent bond between α_2M and proteinase by the thiolester bond loop.


-Gly-NH-CH-CO-Gly-Glu-NH-CH-CO-Asn
CH2
S
C
CH2
CH2
O
PROTEINASE
-NH2
Gly-NH-CH-CO-Gly-Glu-NH-CH-CO-Asn
CH2
SH
CH2
CH2
C=O
HN-
PROTEINASE

The thiol esters of αMs and the complement proteins are located in the highly conserved regions of the molecule (Tack *et al.*, 1980; Campbell *et al.*, 1984; Sottrup-Jensen *et al.*, 1984). Remarkable differences however exist between the reactivities of thiol esters and their function in αMs of different animal species (Table 1.2), thus the access of the thiol ester in mouse and rabbit inhibitor is low and only limited conformational change occurs in murine α_2M in presence of methylamine (Tamamizu *et al.*, 1989; Saito and Sinohara, 1985 a). The thiol esters of rabbit α_2M are less sensitive to methylamine and require very high concentration of the nucleophile for the cleavage of all the four thiol esters. Even in the fully reacted state the inhibitor retains about half of its capacity to inhibit trypsin (Tamamizu *et al.*, 1989). Similarly bovine α_2M (Bjork *et al.*, 1985), rat α_2M (Gonias *et al.*, 1983)and equine α_2M (Motoshima *et al.*, 1988), exhibit only small or no increase in electrophoretic mobility and retain significant proteinase inhibitory activity on methylamine treatment. There exist a marked difference between the human and bovine proteins in amino acid residue side chain size and charge close to thioester bonds. These differences may explain the greater conformational stability of bovine α_2M, subsequent to the cleavage of the thiolester bonds as compared with human α_2M (Bjork and Jornvall, 1986). Other examples of α_2Ms exhibiting comparable sensitivity to methylamine and exhibiting similar alterations in electrophoretic mobility and proteinase inhibition as human α_2M are available in (Tables 1.1 and 1.2).

Green turtle α_2M is unique in that it undergoes polymerization via intemolecular disulphide formation involving sulphydryls released as a result of cleavage of the thiol esters (Osada *et al.*, 1988).

The thiol esters of most αMs exposed to small nucleophiles or proteinases have been considered as "pseudoenzymatic sites "turning over once for all (Sottrup-Jensen, 1987). Pangburn (1992) have however shown that ammonia inactivated α_2Ms, complement C_3 and C_4 regain the thiol esters to a limited extent after removal of excess ammonia. In a more recent study, Gron *et al.* (1996) have shown that ammonia or methylamine induced cleavage of the thiol ester can be reversed at high temperature, and

TABLE 1.2
SENSITIVITY OF THIOL ESTERS OF α-MACROGLOBULINS TOWARDS NUCLEOPHILES

Species	Type	Thiol Ester Cleavage	Conformational Alteration	Exposure of Receptor Recognition	References
Cray Fish	α_2M	Complete	None	n.d.	Hergenhahn *et al.* (1988)
Horseshoe Crab	α_2M	Complete	None	n.d.	Quigley and Armstrong (1985)
Lobster	α_2M	Complete	None	n.d.	Spycher *et al.* (1987)
Plaice	α_2M	Complete	Complete	+	Starkey and Barrett (1982)
Hag Fish	α_2M	Complete	Complete	+	Osada *et al.* (1986)
Salamander	α_1M	Complete	Complete	+	Sallenave and Bellot (1987)
Frog	α_1M	Complete	None	+	Feldman and Pizzo (1986)
	α_2M	Complete	Complete	+	
Turtle	αM	Complete	Complete	+	Osada *et al.* (1988)
	Ovomacroglobulin	Partial	None	n.d.	Osada *et al.* (1988)
Crocodile	Ovomacroglobulin	Partial	None	+	Ikai *et al.* (1983)

Chicken	αM	Compete	None	±	Feldman and Pizzo (1984); Nagase and Harris(1983);
	Ovomacroglobulin	Nil	None	-	Nagase *et al.*, (1983)
Duck	Ovomacroglobulin	Compete	Complete	+	Nagase *et al.* (1986)
Rabbit	α_1M	Complete	Partial	+	Tamamizu *et al.* (1989)
	α_2M	Complete	Partial	+	
Rat	α_1M	Complete	Complete	+	Longberg-Holm *et al.* (1987); Gonias *et al.* (1983).
	α_2M	Partial	None	-	Esnard *et al.* (1985)
	$\alpha_1 I_3$	Complete	None	+	
Mouse	α_2M	Partial	Partial	±	Saito and Sinohara (1985) Gonias *et al.* (1983).
Guinea Pig	α_2M	Complete	None	+	Suzuki and Sinohara (1986)
Horse	α_2M	Complete	None	+	Motoshima *et al.* (1988).
Cow	α_2M	Complete	None	+	Bjork and Jornvall (1986); Dangott and Cunningham (1982); Feldman *et al.* (1984); Bjork *et al.*, (1985)
Pig	α_2M	Complete	n.d.	n.d.	Jacquot- Armand and Guinand (1976); Tsuru *et al.* (1975)
Goat	α_2M	Partial	Partial	n.d.	Khan *et al.* (1999).
Mink	α_2M (Mastitis milk)	Complete	Partial	n.d.	Rantamaki and Muller (1992).

used the procedure to entrap proteins in α_2M without bait region cleavage. Bhattacharjee *et al.* (1999) have demonstrated that under appropriate conditions, mouse α_2M is fully converted to the fast form by NH_3 despite of the aforementioned structural differences between human and mouse α_2M. Thus both molecules are able to incorporate non proteolytic ligands in a similar manner. Ligands incorporated in this manner also demonstrate an enhanced immune response relative to the free ligand.

Inspite of the apparently important role played the proteinases inhibitory property of αMs may not be dependent upon thiol esters *per se* but on their ability to contribute to the characteristics conformation of αMs. Thus while the duck and crocodile ovostatin have conserved characteristic thiol esters, (Nagase, 1986; Ikai *et al.*, 1983) chicken ovomacroglobulin is unique in not containing any (Nagase and Harris, 1983). Surprisingly, however the chicken ovomacroglobulin also undergoes the characteristic proteinases induced "slow" to "fast" transformation (Nagase and Harris, 1983). Other aspects of structure and the mechanism of proteinase inhibition of the egg white protein ovostatin (ovomacroglobulin) are similar to those of plasma α_2M (Feldman and Pizzo, 1984a).

In order to determine the precise role of the thiol esters, Gettins *et al.* (1994) obtained a recombinant variant of α_2M, C949S, expressed in baby hamster kidney cells that lacked the thiol ester forming cysteine. C949S α_2M behaves like methylamine treated plasma α_2M with correctly formed inter-subunit disulphide bridges, but the molecule had exposed receptor recognition site and lacked the ability to inhibit proteinases. Thus correct folding of monomers and their association to the tetrameric form does not require pre-formed thiol esters.

1.2.4 αM receptor/receptor recognition site

The receptor recognition site is one of the three major sites (others are bait region and thiol ester) essential for physiological function of αMs. The αMs attacked by

proteinase(s) or by small nucleophiles such as methylamine undergo a major conformational change resulting in the exposure of a specific receptor recognition site and in the appearance of new antigenic sites, specifically recognized by a monoclonal antibody raised by Marynen *et al.* (1981). Studies of Sottrup-Jensen *et al.* (1985) have shown that the domain bearing the receptor recognition site in human α_2M is also present in complement C_3 and C_4 (Van-Leuven *et al.*, 1986).

The receptor recognition site has been shown to be a part of the proteolytically derived carboxy terminal 20 KDa fragment of human α_2M (Sottrup-Jensen *et al.*, 1986; Van Leuven *et al.*, 1986a;b). Homologous carboxyl-terminal fragments can also be obtained from the tetrameric human α_2M, bovine α_2M and rat α_1M and a monomeric rat α_1I_3 (Enghild *et al.*, 1989). The C-terminal fragments bind to α_2M receptors on peritoneal macrophages and other cells. (Enghild *et al.*, 1989). The low binding affinity of 20 KDa fragment towards the α_2M receptor however suggests that the fragment may not represent the complete receptor recognition site and believed to be a part of a larger 40 kDa C-terminal structural domain (Gordan, 1976; Enghild *et al.*, 1989; Rubenstein *et al.*, 1991; Thomson and Sottrup-Jensen, 1993).

Both human and bovine α_2Ms shows glycosylation at Asn^{1401} (Enghild *et al.*, 1989) 20 KDa fragments. Glycosylation at Asn^{1381}, is seen in bovine but not in the human inhibitor. The overall amino acid sequence identity between the carboxyl terminal 20 kDa domain of human α_2M, bovine α_2M, rat α_1I_3 and α_1M was about 75% (Enghild *et al.*, 1989). Occurrence of two segments of extraordinarily high identity corresponding to residues 1359-1376 and 1424-1435 of the human α_2M sequence suggests that the primary receptor recognition domain may be located in one or both of these region. There exists a Cys^{1329} – Cys^{1444} disulphide bridge in the receptor binding domain of human α_2M which is conserved in bovine α_2M and rat α_1I_3 but not in rat α_1M (Enghild *et al.*, 1989). The interchain disulphide bond is required for binding of the human α_2M carboxyl terminal 20kDa fragment to the receptor (Sottrup-Jensen *et al.*,

1986). In the 20kDa fragment of the human α_2M, this bond, is thought to be necessary to maintain the conformation around the primary receptor recognition site. Enghild *et al.* (1989) proposed that in rat α_1M two phenylalanine residue which substitute for Cys^{1329} and Cys^{1444} may stack, and thus stabilize a conformation in the inhibitor in absence of the disulphides.

The level of circulating αM-proteinase complexes is very low as a result of their rapid clearance primarily by the receptors on hepatocytes (Travis and Salvesen, 1993; Tapon-Bretaudiere *et al.*, 1985; Spolarics *et al.*, 1985). The *in vivo* half life of the complexes is 2–5 mins and they bind with high affinity (Kd = 10^{-8} – 10^{-10} M) to an endocytosing receptor known as α_2M receptor / low density lipoprotein receptor related protein (α_2MR/LRP) (Van-Leuven *et al.*, 1986). The receptors have been identified on fibroblast cells like, monocytes / macrophages and syncytiotrophoblasts (Tapon–Bretaudiere *et al.*, 1985; Van-Leuven *et al.*, 1986, Sottrup-Jensen *et al.*, 1986, Pizzo, 1988; Jensen *et al.*, 1988).

The Kd of human and bovine receptor binding domain to α_2MR/LRP (~60-125 nM) in different systems (Van Leuven *et al.*, 1988; Sottrup-Jensen *et al.*, 1986; Enghild *et al.*, 1989) is greater than the Kd for binding α_2M-methylamine (~ 40 pM and 2 nM for the high and low affinity binding respectively) (Moestrup and Gliemann, 1991). Therefore, it has been questioned whether receptor binding domain in its structure contains all information necessary for receptor recognition by α_2M (Enghild *et al.*, 1989; Gliemann *et al.*, 1995). However, the finding that human receptor binding domain variant (RBDv), which contains 12 additional upstream residues, binds to α_2MR/LRP with a Kd of ~8 nM indicates that C-terminal fragment may indeed constitute the receptor binding domain of α_2M, but its conformation may be subtly affected by neighbouring residues (Hotlet *et al.*, 1994). Receptor binding may principally involve only one receptor recognition domain and is Ca^{2+} dependent. (Gliemann and Sottrup-Jensen, 1987). The sequence of internalization and degradation of αM–proteinase complexes may be analogous with that of the low density lipoprotein (Gliemann *et al.*, 1989).

Functionally active α_2M receptor (α_2MR) has been purified from human placenta, rat hepatocytes and human fibroblasts (Aschom *et al.*, 1990; Jensen and Pizzo, 1989). The receptors do not discriminate among various mammalian αMs (Sottrup-Jensen, 1987; Gliemann and Sottrup-Jensen, 1987). However that the mammalian receptors do not recognize the ovostatins (Feldman and Pizzo, 1984b). Sequence analysis of purified human placental α_2M receptor indicates that it is identical to LRP (Strickland *et al.*, 1990; Kristensen *et al.*, 1990; Jensen et al., 1989). This receptor is of a classic scavenger type with a single membrane-spanning domain that binds a variety of ligands including the Pseudomonas exotoxin A, plasminogen activator, lactoferrin and lipoprotein lipase (Kounnas *et al.*, 1992; Nykjaer *et al.*, 1993; Orth *et al.*, 1992; Bu *et al.*, 1992; Chappel *et al.*, 1992; Willnow *et al.*, 1992). These ligands do not normally compete with each other for binding to LRP/α_2MR, presumably because they are recognized by independent sites (Krieger and Herz, 1994).

Misra *et al.* (1993) identified a second α_2M receptor, a G-protein coupled α_2M receptor, distinct from the LRP/α_2M receptor. On ligation this receptor induces a signalling cascade resulting in the production of myo-inositol 1,4,5–triphosphate (IP_3) and an increase in intracellular Ca^{2+} and cyclic AMP. Ligation of α_2M-methylamine (α_2M-$MeNH_2$) or receptor binding fragment (RBF) from rat α_1M induces phosphorylation of a number of proteins including phospholipase Cγ1 (Misra *et al.*, 1994; 1995).

The binding of human α_2M-methylamine to its receptors on macrophages has also been shown to regulate tumour kill, the respiratory bursts, prostaglandin synthesis and secretion of proteinases (Johnson *et al.*, 1982; Hofmann *et al.*, 1983; Uhing *et al.*, 1991). During the proteinase attack on the inhibitor, α_2M can incorporate antigens and augment macrophage-dependent antigen presentation to T-cells (Chu and Pizzo, 1993).

1.2.5 Transglutaminase reactive site

Transglutaminases (TGs) are abundantly produced by numerous cell types including macrophages, keratinocytes and endothelial cells and α_2M is the major TG substrate in human serum. (Van Leuven, 1984). The site of transglutamines reactivity is located approximately 20 amino acids upstream from the primary proteinase cleavage site in the bait region (Sottrup-Jensen, 1989). TGs catalyse the incorporation of primary amine and peptide into the TG reactive sites of α_2M. (Borth, 1992). TG reactive site is also involved in the clustering of membrane bound α_2M-proteinase complex and its endocytosis in a variety of cells (Imber and Pizzo, 1981; Mortensen *et al.*, 1981).

1.2.6 Carbohydrate chain

Alpha-2-macroglobulins from all the investigated sources are glycosylated to varying extents. Dunn and Spiro (1967) reported that carbohydrates make up 9.43% of the total dry weight of human α_2M. Pronase digest showed that human α_2M contained 31 carbohydrate chains per molecule with an average of 3 residues of mannose, 2 of galactose, 4.7 of N-acetylglucosamine, 1.5 of sialic acid and 0.4 of fucose. The carbohydrate unit is found attached to the polypeptide by glycosylamine linkage to the asparagyl residue (Dunn and Spiro, 1967). Two points of attachment of these oligosaccharide have been identified, one is the thiol ester bond loop region and other is the 20 KDa receptor binding domain (Enghild *et al.*, 1989).

Rat α_1M and α_2M have almost similar carbohydrate content and are in close agreement with the results obtained from a number of other species (Gauthier and Mouray, 1976). Jamieson *et a.* (1972) however, obtained a higher hexose and sialic acid content in rat α_2M as compared with other α_2Ms. α_2M from bovine and hamster contains nearly 10% by weight of asparagines linked carbohydrate as is the case of human PZP (Nagasawa *et al.*, 1970; Southard and Talamantes, 1989).

Chicken αM and ovomacroglobulin terminates in galactose which mediates the clearance of αM-complexes by receptors present on various cell types whereas 4-0 acetylated sialic acid is uniquely present on equine α_2M (Prichitt and Paulson, 1989). The oligosaccharide structures of human, equine and guinea pig α_2M has provided the complete structural information on the N-linked sugar chains of this plasma glycoprotein (Hanaoka *et al.*, 1989).

The component sugar analysis of limulus α_2M showed the existence of a complex type of oligosaccharide side chain similar to those of mammalian α_2M. However, unlike mammalian α_2M, no sialic acid was detected in limulus α_2M and it contained 3 mol/ mol N-acetylglucosamine, suggesting the presence of O-linked sugar chains which have not been found in mammalian α_2M (Armstrong et al., 1991).

1.3 Conformational changes

When tetrameric αMs are incubated with proteinases or small molecular weight amines their conformation undergoes remarkable alteration. These were originally characterized as change from the electrophoretically "slow" –S from to "fast" –F form. A variety of other physical methods have been used subsequently to follow the proteinase and amine induced conformational changes like hydrodynamic techniques (Gonias *et al.*, 1982; Bjork and Fish, 1982) and small x-ray scattering (Branegaard *et al.*, 1982; Osterberg and Malmester, 1984). Evidence from electron microscopy also revealed the remarkable reorganization of tetrameric αMs (Sottrup-Jensen, 1989).

Proteinase and amine induced conformational changes in dimeric and monomeric αMs are less pronounced, but have been revealed by immunochemical procedures (Delain *et al.*, 1988; Carlesson-Bostedt *et al.*, 1988), UV-difference spectroscopy, circular dichroism and fluorescence (Sottrup-Jensen, 1987).

A complex interplay between bait region cleavage and cleavage of the thiol ester

appears to contribute the conformational state of αMs. One consequence of the proteinase and amine induced conformational changes is the exposure of receptor recognition site on the molecule which allows rapid clearance of "fast" form from the circulation by a variety of cells (Van Leuven *et al.*, 1979). Although receptor recognition site is exposed both in proteinases and amine induced form of most αMs, but the resulting conformation alteration differs subtly (Delain *et al.*, 1988) and mechanisms involved may vary for αMs from various species. Interaction of equine α_2M with proteinases and methylamine resulted in clearly more than one conformational change as studied by circular dichroism and electrophoresis (Lah *et al.*, 1987; Motoshima *et al.*, 1988). Table 1.2 summarizes the receptor recognition site exposure after amine treatment in many species.

In rat and rabbit α_2M the amine induced thiol ester cleavage results in receptor recognition site exposure, but the characteristic large conformational change is only elicited subsequent to the bait region cleavage by proteinases (Longberg-Holm *et al.*, 1987; Tamamizu *et al.*, 1989). Bovine and equine α_2Ms do not display any difference in electrophoretic mobility between native and methylamine treated forms even though only the latter is receptor recognized (Bjork *et al.*, 1985; Bjork and Jornvall, 1986 Motoshima *et al.*, 1988). Spectroscopic studies also show that bovine α_2M is altered only minimally by methylamine exposure consistent with no major change in secondary and tertiary structure of the protein (Bjork *et al.*, 1989). In PZP and rat α_1I_3 also the large conformation change and full exposure of the receptor recognition site is only achieved in the methylamine induced form by subsequent bait region cleavage by proteinases (Table 1.2) (Carlsson–Bostedt *et al.*, 1998; Gliemann and Sottrup-Jensen, 1987).

Longberg-Holm *et al.* (1987) have observed by electron microscopy the conversion of globular ring shaped native forms of human and rat α_1M to rectangular box like structures on proteinase treatment. Electron micrographs of rat α_1M and α_2M revealed compact and irregular shapes on proteinase interaction

The migration behaviour in polyacrylamide gel electrophoresis of the dimeric αM from plaice remains unaltered after treatment with proteinase (Starkey and Barrett, 1982). Unlike other dimeric αMs, limulus αM exhibit characteristic "slow" to "fast" transformation in electrophoresis (Quigley *et al.*, 1991). The monomeric rat α_1I_3 showed no alteration in electrophoretic mobility on reaction with methylamine (Longberg Holm *et al.*, 1989).

The trigger for the conformational change appears to be the cleavage at different positions within a large span of unique bait region sequence among the various αMs (Sottrup Jensen, 1989). Presumably, a profound reorientation of several domains occurs as a result of activation consistent with the findings that bait region cleavage of human α_2M extensively crosslinked by cis-dichloro diammine platinum (II) does not result in thiol ester activation unless the crosslinks are subsequently removed (Roche *et al.*, 1989). Much however seems to remain in the understanding of the conformational changes associated with thiol ester cleavage.

1.4 Shapes and models of αMs

Numerous studies on the shapes of native and transformed α_2Ms are available. Native αMs frequently appear as irregular objects suggesting a slender structure (Longberg-Holm *et al.*, 1987 Ruben *et al.*, 1988), or shaped like doughnuts (Tapon-Bretaudiere *et al.*, 1985; Ikai *et al.*, 1987), padlocks (Delain *et al.*, 1988; Tapon-Bretaudiere *et al.*, 1985; Bergsma *et al.*, 1985; Bretaudiere *et al.*, 1988; Ikai *et al.*, 1987) or like assemblies of four or five globules (Delain *et al.*, 1988; Bergsma *et al.*, 1985; Ikai *et al.*, 1987). It has recently been shown that all these shapes correspond to one single structure (Larquet *et al.*, 1994). In a three dimensional reconstruction of native human α_2M the dimensions of the molecule were estimated to be 100 and 140 °A. In contrast electron micrographs of "fast" form obtained from human and other tetrameric α_2M reveal a well defined monogram like objects resembling the Cyrillic letter referred to as the)-(View (Bloth *et al.*, 1968). Its dimensions as estimated from projections of

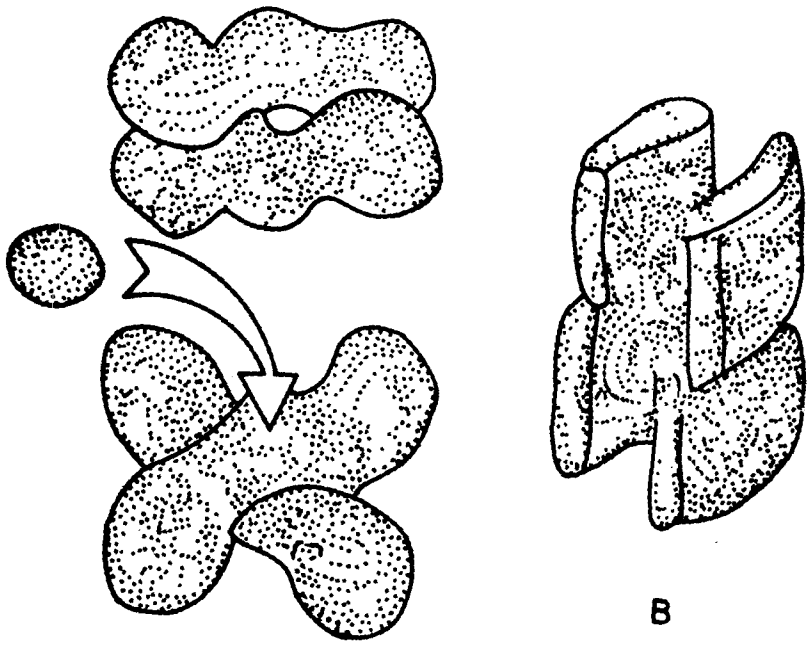
different orientations and from three-dimensional reconstructions are 180-200 °A, 120–140 °A and 80-90 °A (Tapon-Bretaudiere *et al.*, 1985; Bretaudiere *et al.*, 1988; Delain *et al.*, 1992). Receptor recognition sites are located at the tip of the arms of the)-((Delain *et al.*, 1988; Hussaini *et al.*, 1990). Molecules probably representing intermediates in the transition from native to transformed α_2Ms have also been observed (Delain *et al.*, 1992; Gonias and Figler, 1989; Marshall *et al.*, 1992).

The proteinase(s) in the α_2M-proteinase complex appear to partially fill a large elongated cavity (Delain *et al.*, 1988; Boisset *et al.*, 1989; Boisset *et al.*, 1992; Schrocter *et al.*, 1992) which seems to be empty in α_2M-methylamine (Boisset *et al.*, 1989; Boisset *et al.*, 1992a;b; Schroeter *et al.*, 1992). The flexible bait regions are relatively close to the thiol esters (Gettins *et al.*, 1988; Gettins *et al.*, 1990). The latter are probably located in the centre of the molecule at the inner surface of the cavity (Boisset *et al.*, 1992 a & b: Boisset *et al.*, 1994). Hydrodynamic data indicate an elongated shape of active dimers obtained by urea treatment (Liu *et al.*, 1987; Roche *et al.*, 1988a;b) whereas dimers formed by Zn^{2+} or Cd^{2+} appear as curved structures or rounded rods in which three globules can be seen (Delain *et al.*, 1988; Thomas *et al.*, 1988). In an electron microscopic study of chymotrypsin treated rat α_1I_3, the molecule appears squarish with good contacts between the front and back parts and a well-defined internal cavity (Ikai *et al.*, 1990).

Several models differing in symmetry and localization of disulphide – bridged dimers have appeared (Liu *et al.*, 1987; Bretaudiere *et al.*, 1988; Delain *et al.*, 1988; Feldman *et al.*, 1985; Andersen *et al.*, 1995).

The model shown in Fig. 1.5 by Delain *et al.* (1988) proposes that in native tetrameric human α_2M two rod like disulphide-bridged dimers are assembled to form a cross-like structure. This model accounts for the generally elongated shape of the dimers and monomers and emphasizes that in native state the bait region segments are highly

Figure 1.5 Model of human α_2M proposed by Delain *et al.* (1988) showing native (A)and proteinase transformed (B) α_2M.


A
B

accessible to proteinases. The activated thiol esters face the lumen of the cavities positioned in close proximity to the surface of the trapped proteinases.

1.5 Role of metal ions

α_2M functions as the major metal binding protein of human plasma. A number of metal ions have been shown to bind to α_2M including cadmium (Watkins *et al.*, 1982; Scott and Bradwell, 1983), calcium and magnesium (Dumitrescu *et al.*, 1977), nickel (Nomoto *et al.*, 1982) and zinc (Parisi and Vallee, 1970; Prasad and Oberleas, 1990). According to Adham *et al.* (1977), there are two classes of binding sites for zinc on α_2M, the high affinity sites which bind 12g-atom of Zn^{2+} /mole of α_2M protomer and the low affinity sites that bind upto 60 g-atom of Zn^{2+}. The reported dissociation constant (Kd) 3.27 x 10^{-8}M by Adham *et al.* (1977) agrees with the subsequent findings of Pratt and Pizzo (1984) which proposes a Kd value of $8x10^{-7}$M. Likewise, neither saturation on all the high affinity sites with Zn^{2+} nor removal of all the zinc from α_2M had any effect on the trypsin binding activity. However, saturation of the low affinity sites led to a remarkable decrease in trypsin binding activity which was ascribed to irreversible aggregation of α_2M (Pratt and Pizzo, 1984).

The distribution of zinc between α_2M and other serum zinc binding proteins has been studied to assess the role of the inhibitor in zinc metabolism in health and disease (Falchuk,1977; Song and Adham, 1979). Gettins and Cunningham (1986) reported that the relative affinity of zinc for native and methylamine modified form of α_2M is different. Whereas, the affinity for Cd^{2+} of the methylamine modified and native forms were comparable, far fewer bindings sites were available in the former (Carson, 1984). They reported that α_2M bound 30-40% of the total serum zinc. Pratt and Pizzo (1984) reported that at physiological human serum zinc concentration (10-15 μM), α_2M appears to bind 20 mole zinc / mole. Zinc bound to high affinity sites on α_2M may be delivered to various tissues when the α_2M-proteinase complexes are endocytosed (Maxfield *et al.*, 1978).

The structural and functional implications of zinc binding to α_2M at high concentration of zinc, while not physiologically relevant, are useful as probes of protein-metal interactions, particularly in relation to subunit association. The absence of profound effects on α_2M structure or function at physiological zinc concentrations indicates that α_2M may play a role in zinc transport *in vivo* (Pratt and Pizzo, 1984).

Couture-Tosi *et al.* (1986) observed that at low temperatures the dimers formed from Zn^{2+} reassociate in long linear polymers which display a regular chain like arrangement and a helical periodicity.

Carson (1984) carried out the interaction of Cd^{2+} with α_2M and proposed a model that α_2M binds Cd^{2+} with relatively high affinity and with a stoichiometry consistent with a single binding site per protein subunit. Occupancy of all measured sites by Cd^{2+} parallels a small increase in the apparent ability of α_2M to protect trypsin from inactivation by soyabean trypsin inhibitor. Later Pochon *et al.* (1987) reported the dissociation of α_2M into dimeric units by Cd^{2+}. This dissociation is consistent with the presence of the binding sites for divalent cations at the contact surface of two α_2M dimeric units. A detailed characterization of these binding sites, was not possible due to the aggregation of α_2M above $1x10^{-7}$M Cd^{2+} (Gettins and Cunninghams, 1986).

1.6 Biological functions

Surprisingly, while the α_2Ms have been implicated in large variety of biological functions, their exact physiological roles continues to remain elusive. In view of their broad specificity in inhibiting proteinases they may have a general role in restricting or controlling proteolysis. Indeed in evolutionary terms they have been regarded as a part of a primitive defence mechanism against harmful intrinsic/extrinsic proteolytic enzymes (Quigley and Armstrong, 1983a). Besides controlling proteinases in concert with other inhibitors, they may function as backup inhibitors under conditions where primary inhibitors eventually become depleted, e.g. in clotting and fibrinolysis (Heumann and

Vischer, 1988; Petersen *et al.*, 1989). α_2M may further bind proteinases dissociated from reversible proteinase – inhibitor complexes (Heumann and Vischer, 1988).

Target proteinases for α_2M may be found among those associated with ovulation (Gaddy-Kurten and Richards, 1991), matrix remodelling (Enghild *et al,* 1989), snake bites (Baramova *et al.,* 1990), microbial infections (Miyoshi and Shinoda, 1991), viral infections (Prichett and Paulson, 1989) and larval infection (Bowles and Feehan, 1990). Though α_2M is not an acute phase reactant in humans and even partial deficiency is rare, the induction of synthesis of PZP during pregnancy in humans and α_2M in the rat during inflammatory state suggests that these αMs may augment the existing potential for proteinase clearance under such conditions (Chaudhari, 1993; Webb *et al.,* 1995). In rat, α_2M probably has a predominant role, compared with human α_2M as a TGF-β, carrier in the plasma during the acute phase response. Rabbit α_2M, an acute phase reactant has been found to offer protection against corneal ulceration. (Berman *et al,* 1975).

The occurrence of α_2M complexes in plasma with severe illness viz acute pancreatitis (Donnelly *et al.,*1983; Larson & Ohlsson,1984), leukaemia and metastatic melanoma (Hubbard, 1983) has earlier been reported from different laboratories. Bohn *et al.* (1980) reported that high doses of α_2M are required to inhibit proteinase which plays a positive role in the transformation hence the clinical significance of α_2M cannot be ruled out.

The cleavage of thiol ester as a means of activating α_2Ms probably reflects the concept that this form of α_2M acquires new function, reminiscent of a binding, transporting and targeting protein that can modulate biological response. α_2M has been identified as a major plasma binder and carrier for several important cytokines. These includes transforming growth factor β-1 and β-2 (TGFβ-1 and β-2) (Daniel pour and Sporn, 1990; Huang *et al.,* 1988; LaMarre *et al.,* 1998), platelet derived growth factor (PDGF) (Bonner *et al.,* 1990; Bonner *et al.,* 1989), basic fibroblast growth factor (Dennis

et al., 1989), interleukin – 6 (IL-6) (Matsuda *et al.*, 1989). The conformation of α_2M also appears to influence the affinity for cytokines. Human α_2M in native state binds PDGF (Bonner *et al.*, 1990; Bonner *et al.* 1989). TGF-β_2 (Daniel Pour and Sporm 1990; LaMarre *et al.*, 1990), TGF β_1(Huang *et al.*, 1988) and IL-6 (Matsuda *et al.*, 1989). Some cytokines that weakly or not at all bind to native α_2M demonstrate preferential binding to "fast" form of α_2M (LaMarre *et al.*, 1991). TGBβ_1 and TGFβ_2 bind preferentially to methylamine treated α_2M (Huang *et al.*, 1988). Similarly, tumour necrosis factor (TNF-α) and IL-2 binds to "fast" form of α_2M (La Marre *et al.*, 1991; Borth and Luger, 1989). The nature of binding of cytokine varies with different cytokines. It could be non covalent as in case of TNFα and TNF-β (Huang *et al.*, 1988) or it could be thiol ester dependent mechanism as of IL-2 (Legres *et al.*, 1995). The binding between cytokine and α_2M could be the result of thiol disulphide interchange as with IL-2 (Legres *et al.*, 1995) or could be other type viz TGF β/α_2M complex which can be dissociated by heparin (McCaffery *et al.*, 1989). By functioning as cytokine carrier, native α_2M may lengthen plasma life of bound cytokine and the α_2M-cytokine complexes may be dissociated by proteinases (Matsuda *et al.*, 1998), low pH (Huang *et al.*, 1988) or heparin (LaMarre *et al.*, 1990) normally present near the cell surface. The α_2M cytokine complex are cleared *in vivo* via the α_2M receptor (LaMarre *et al.*, 1991).

The αMs have a unique ability to bind a wide variety of other molecules and ions such as soluble Ia antigens, histones, encephalitogenic protein, zinc, nickel, lithium carmine, liposomes and immune complexes (James, 1980). Human α_2M is a major zinc binder and carrier protein (Parisi and Vallee, 1970). Zinc is not required for proteinase binding function of α_2M, but may be essential for the binding of cytokines such as IL-β (Borth and Luger, 1989; Borth *et al.*, 1990).

Other specific roles for α_2M have also been suggested. For instance a study has indicated the potential of α_2M in reducing harmful free radical production (Donnelly *et al.*, 1991). α_2M have implicated in modulating proteotylic cleavage of various peptide

hormones (Rinderknecht and Geokas, 1973). Other evidence suggest that α_2M is important in modulating various immune responses as well as in inflammatory response (James, 1980). Hubbard *et al.* (1987) have shown that α_2M acts as a immuno suppressive agent in its proteinase complex form.

Experimental

2.0 MATERIALS

Chemicals and reagents used in this study were obtained from the following sources as details below. Glass distilled water was used in all the experiments.

B.D.H. Poole, England.

Bromophenol blue.

B.D.H. India.

Copper sulphate, Ethanolamine, Ethylenediamine, tetraacetic acid, Hydrochloric acid, Trichloroacetic acid, Phenol.

C.D.H. India.

Sodium thiocyanate.

Difco Laboratories, U.S.A.

Freund's Complete Adjuvant, Freunds incomplete adjuvant.

E. Merck., India.

Ammonium sulphate, Dithiozone, Glycine, Methanol, Sodium hydroxide, Sodium Potassium tartarate.

E. Merck, Germany.

Pronase E, Proteinase K.

Genei Pvt. Ltd., Banglore.

High molecular weight marker.

Koch-light Lab. Ltd., England.

N,N,N',N' – tetramethylene diamine.

Merck-Scheveherdt., Germany.

β-mercaptoethanol.

Pharmacia Fine Chem., Sweden.

Blue dextran, Sephadex G-200, Sephacryl S-300 HR, Sepharose–4B.

Qualigens, India.

Acetic acid, acetonitrile, Di-sodium hydrogen phosphate, glycerol isopropanol, Di-hydrogen sodium phosphate, potassium iodide, potassium chloride, sulphuric acid, sodium chloride, sodium bicarbonate, sodium carbonate, zinc chloride, glucose, methylamine.

Sisco Res. Lab., India.

N,N'-methylene biscacrylamide, acrylamide, ammonium persulphate, bovine serum albumin, chymotrysin, casein, cyanogen, bromide, Folins ciocalteu's phenol reagent, glutathione, N,N'–Dithio (bis) nitrobenzoic acid, sucrose, sodium citarate, trypsin.

Sigma Chemical Co., U.S.A.

Agarose, apoferritin (Horse Spleen), DL-Benzoyl arginine p-nitroanilide, coomassie brilliant blue R-250, β-galactosidase, phenyl methyl sulphonyl fluride, sodium dodecyl sulphate, soyabean trypsin inhibitor, thyroglobuliun (Bovine), Tris (hydroxymethyl) amino methane, ovalbumin, Hemoglobin.

Sarabai Chemicals, India.

Dimethyl sulphoxide.

3.0 METHODS

3.1 Purification of buffalo blood alpha-2-macroglobulin

Fresh buffalo blood was collected from the abbatoir immediately after slaughter into bottles containing 1.32% (w/v) sodium citrate and 1mM PMSF/SBTI. The plasma separated from the cells by centrifugation at 750 g was collected.

3.1.1 Ammonium sulphate fractionation

One hundred millilitre of buffalo plasma was 20% saturated with ammonium sulphate with gentle stirring at 4°C. After 4-6 h, the precipitate was removed by centrifugation at1750 g for 15 min. and the supernatant collected was made 40% saturated with ammonium sulphate. The solution was allowed to stand at 4-6^{0}C for 8 h, the precipitate recovered by centrifugation at1750 g for 20 min and dissolved in 20 ml of distilled water. The fraction was extensively dialysed against several changes of ice-cold buffer (0.1 M sodium phosphate buffer containing 0.1 M KCl, pH 8.0).

3.1.2 Gel filtration chromatography

A Sephadex G-200 column was prepared as recommended by Peterson and Sober (1962). The gel was allowed to swell in sufficient amount of distilled water for 6 h in a boiling water bath. The gel fines were removed by suspending the gel in two to four fold excess 0.1M sodium phosphate buffer, pH 8.0 and allowing 90-95 % of the gel to settle down. The remaining gel in supernatant was rapidly removed by suction. A glass column mounted on a sturdy vertical support was filled to one third of its length with operating buffer in order to check leaks and flush air bubbles from the dead space. The deaerated gel slurry was then poured with a help of glass rod into the column with care to avoid generating air bubbles. The column was left standing overnight. Flow rate was increased gradually and after accomplishing a constant flow rate higher than that required for final elution the column was adjusted to the required flow rate. The packed column was thoroughly washed with two bed volumes of operating buffer (0.1M sodium phosphate buffer containing 0.1M KCl, pH 8.0). In order to check uniform packing and to determine

the void volume of the column, 2% (w/v) solution of blue dextran in 0.1 M sodium phosphate buffer, pH 8.0 was passed through the column. The volume of blue dextran and protein solution applied was not more than 2–3% of the total bed volume. Three millilitre fractions were collected and assayed for protein and α_2M activity. Homogenity of the preparation was analyzed by 5% PAGE.

3.2 Colorimetric Analysis

3.2.1 Determination of protein concentration

Protein was estimated by the method of Lowry *et al.* (1951). Aliquots of protein solution were taken in a set of tubes and final volume was made upto 1 ml with 0.01 M sodium phosphate buffer, pH 8.0. Five ml of alkaline copper reagent (containing one part of 0.5%(w/v) copper sulphate in 1%(w/v) sodium potassium tartarate and 50 parts of 2%(w/v) sodium hydroxide) was added followed after 10 min of incubation at room temperature, with 0.5 ml of 1.0N Folins–Ciocalteu's phenol reagent. The tubes were instantly vortexed. The colour developed was read at 660 nm after 30 min against a reagent blank. A standard curve was prepared using BSA as standard. Protein in the column fractions was monitored at 280 nm in Beckman DU-40.

3.2.2 Assays of α_2M activity

α_2M activity was quantitated either by its ability to inhibit proteolytic cleavage of casein by trypsin (Kunitz,1947) or trypsin esterase activity described by Ganrot (1966).

Inhibitory assay of α_2M was performed as described by Kunitz (1947). Buffalo α_2M was examined for its ability to prevent various proteinase(s) from digesting casein. For determination of inhibitory activity, α_2M proteinase(s) complex was incubated with casein for 15 min in 0.1 M sodium phosphate buffer, pH 8.0 and reaction stopped by the addition of 20% TCA. Acid insoluble material was removed by centrifugation at 1750 g for 15 min. The supernatant was analyzed for acid soluble peptides with Folin's phenol reagent as described by Lowry *et al.* (1951).

α_2M activity was also measured by its ability to protect trypsin esterase activity (TPE-activity) from SBTI, as described by Ganrot (1966). Appropriate amounts of the inhibitor were diluted to 1.0 ml with 0.01 M sodium phosphate buffer, pH 8.0. To this was added 0.1 ml of 0.1% trypsin (prepared in 0.0025 N HCl). After incubation at 37°C for 10 min, 0.1 ml of 0.1% SBTI (prepared in 0.0025N HCl) was added and the sample incubated for further 10 min. Two millilitre of freshly prepared BAPNA solution (prepared by dissolving 43.5 mg BAPNA in 1.0 ml DMSO and then the solution was brought to 100 ml with phosphate buffer) was added to the reaction mixture and the final volume to made 3.0 ml with the buffer after which samples were incubated simultaneously. The reaction was stopped by addition of 0.5 ml of 30% acetic acid and the absorbance of the yellow coloured p-nitroanilide formed was determined at 410 nm. The amount of substrate hydrolyzed by the enzyme was calculated using molar extinction coefficient of 8800M $^{-1}$cm^{-1} for p-nitroanilide at 410 nm.

3.2.3 Carbohydrate estimation

The procedure described by Dubois (1956) was followed. Two millilitre aliquots containing 10 to 70 µg of carbohydrate was pipetted into a test tube and 0.05 ml of 80% phenol added. This was followed by the addition of 0.5 ml of concentrated sulphuric acid, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min. thoroughly mixed and again incubated for 20 min. at 30°C. The colour intensity was measured at 490 nm for the quantitation of hexose content. Glucose was used as the standard.

3.2.4 Zinc estimation

Zinc estimation in plasma and α_2M was performed as described by Song *et al.* (1976). To 1.0 ml aliquot containing 0.03 to 5 µg zinc, 0.1 ml of dithiozone solution (prepared by dissolving 1mg dithiozone in 1.0ml of 1M Tris buffer, pH11) was added and after mixing the absorbance was read at 555 nm in Spectronic-21 spectrophotometer.

Two blanks were prepared, one containing distilled water and dithiozone, the other containing zinc but no dithiozone. The blank readings were subtracted from the absorbance of the samples being assayed and zinc content determined from the standard graph. Appropriate aliquots of α_2M were also analyzed similarly for the zinc content.

To 3 ml of plasma in a plastic tube 30 mg potassium iodide was added, and after mixing 0.15 ml of 100% TCA was added. The sample was then shaken, allowed to stand at room temperature for 10 min, and centrifuged at 3,600 g for 30 min.The pH of the supernatant was adjusted to approximately 13.5 by adding 0.1 ml, 10 M NaOH per 1.0 ml supernatant. The mixture was allowed to stand for 15min and centrifuged as above. The precipitate $(CaOH)_2$, $(MgOH)_2$, $(CoOH)_2$, $(MnOH)_2$ and $(FeOH)_2$ was discarded and to 0.8 ml of the supernatant, 0.1 ml of 6 N HCl and 0.1 ml of saturated Tris buffer were then added in this order, followed by 0.1 ml dithizone reagent in 1M Tris buffer. The pH of this mixture prior to addition of saturated Tris buffer remained between 1 and 8.5. Absorbance was determined at 555 nm.

3.2.5 Thiol group estimation

The procedure described by Ellman (1959) was followed for the estimation the thiol groups of α_2M. Trypsin and methylamine induced appearance of free thiol groups in the α_2M was followed by titration with DTNB reagent. Appropriate aliquots of 0.5 ml, native, trypsin or methylamine treated α_2M were mixed with 0.1 ml of DTNB reagent (prepared by dissolving 80 mg in 0.2 ml of 0.05 M Tris-EDTA buffer, pH 8.0) in a total volume of 3.1 ml. The absorbance was read after 15 min at 412 nm in Beckman DU-40 recording spectrophotometer. Free thiol concentration was calculated from the absorbance, using molar extinction coefficient of 13,600 M^{-1} cm^{-1} for the thionitrobenzoic acid group released. A standard plot was prepared using glutathione.

3.3 Preparation of modified α_2M

3.3.1 Treatment with methylamine

Appropriate amount of α_2M was incubated with 0.5 M methylamine in 0.1M sodium phosphate buffer, pH 8.0 at 22°C for 24 h. The protein was then extensively dialysed against 0.1M sodium phosphate buffer, pH 8.0 (Imber and Pizzo, 1981).

3.3.2 Treatment with trypsin

Appropriate aliquots of α_2M were incubated with 2-fold molar excess of trypsin at 37°C for 15 min after which the proteolysis was inhibited with a 100-fold molar excess of PMSF. Alternatively, α_2M was also treated with three-fold molar excess of immobilized trypsin for 3 h at 37°C. The inhibitor was separated from the immobilized trypsin by centrifugation.

3.4 Slab Gel Electrophoresis

3.4.1 Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed by the Tris-glycine system of Laemmli (1970) using the apparatus manufactured by Biotech, India. Concentrated stock solution of 30% acrylamide containing 0.8% bisacrylamide and 1.5 M Tris pH 8.8 were mixed in appropriate proportions to the required final concentration. It was then poured into the mould formed by two glass plate (8.5x10 cm) separated by 1.5 mm thick spacers. Bubbles and leaks were avoided A comb providing a template for 7 samples wells were inserted into the stacking gel solution before polymerisation began. The polymerisation was complete in about 30 min. after which the comb was removed and wells overlaid with the running buffer. Routinely, a 5% acrylamide gel was used. Samples containing 10-20 µg of protein were prepared to give a final concentration of 62.5 mM Tris-HCl pH 6.8, 10%(v/v) glycerol and 0.001% bromophenol blue as a tracking dye.

3.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was essentially performed by the method of Laemmli (1970). Concentrated stock solutions were mixed in the appropriate order to give a final concentration of 7.0%. Protein samples were prepared in solution containing of 62.5 mM

Tris-HCl pH 6.8, 10%(v/v) glycerol, 2%(w/v)SDS, 5%(v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue. The samples were incubated at 37^0C for 45min. Electrophoresis was performed at 100V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192 mM glycine and 25 mM Tris-HCl pH 8.8.

3.4.3 Coomassie Blue Staining

After electrophoresis, the gels were stained with five gel volumes of 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid for at least 4 h. For destaining the gels were incubated with shaking in 5% methanol and 7.5% acetic acid at room temperature.

3.5 Molecular weight determination

The molecular weight of buffalo α_2M was determined under native and denaturing conditions by gel filtration and SDS-PAGE respectively.

The molecular weight of buffalo α_2M and α_2M half molecule formed by urea was computed from its elution volume on a Sephacryl S-300 HR column (2.5x 85 cm).The column was calibrated by determining the elution volume of several globular proteins-thyroglobulin (668 KDa), Appoferritin (440 KDa), β-galactosidase (116 KDa), Hemoglobin (64 KDa) and Ovalbumin (45 KDa). The data was analysed according to the theoretical treatment of Andrews(1964).The linear plot between Ve/Vo and log M was used in calculating the molecular weight of buffalo α_2M and its half molecule.

Subunits molecular weight of α_2M was calculated by the procedure of Weber and Osborn (1969) using SDS-PAGE. The mobilities of marker proteins determined under identical condition were plotted against the logarithm of molecular weight. The standard protein used were myosin (205 KDa), phosphorylase (97 KDa), bovine serum albumin (68 KDa), ovalbumin (43 KDa) and carbonic anhydrase (29 KDa). The analysis of data

indicated a linear relationship between Log M and relative mobility (Rm) and the plot was used in calculating the subunit molecular weight of α_2M.

3.6 Determination of Stokes radius

Size exclusion chromatography on Sephacryl S-300 HR (2.5 x 85 cm) column equilibrated with 0.1 M sodium phosphate buffer, pH 8.0 containing 0.1 M KCl, was used to determine the Stokes radius of α_2M and α_2M half molecules. The flow rate was maintained at 50 ml h^{-1} and the absorbance of the eluent was monitored at 280 nm. The Stokes radius of α_2M and its half molecule was determined by the method of Laurent and Killander (1964) using gel filtration data. The standard proteins used were thyroglobulin (86°A), Apoferritin (59°A), catalase (52°A), aldolase (46°A) and bovine serum albumin (35.5°A). The value of Kav of each marker protein was calculated from the formula

$$Kav = Ve - Vo / Vt - Vo$$

By substituting the values of (Ve elution volume)

Vo (void volume)

Vt (total volume of column)

A graph of the square root of the negative logarithm of Kav versus stokes radius gave a linear plot which was used in calculating stokes radius of buffalo α_2M and α_2M half molecule.

3.7 Amino acid analysis

Protein (500 µg) was hydrolysed with 6 N HCl at 110^0C in sealed ampoules. The digested sample were redried in a mixture of ethanol, water and TEA in a ratio of 2:2:1 in vacuo. This was followed by derivitization of amino acids. A mixture of ethanol, TEA, water and PITC in a ratio of 7:1:1:1 was added to α_2M sample at 22^0C and left for 20 min. Again the sample was dried in vacuo and resuspended in sample diluant which was filtered by passing through sample clearance filter (0.25 µm). A 40 µl of sample was then finally injected in automatic amino acid analyser.

Peak of respective amino acid spectra was worked out matching with standard amino acid peaks. Concentration of each amino acid was calculated by measuring the area of the respective peak. Number of amino acid in the protein were calculated by dividing the concentrations of each amino acid by total concentration of amino acid in the protein and multiplying by total number of amino acid residues in the protein. On hydrolysis, asparganine and glutamine are converted to aspartic and glutamic acid and hence were calculated together with those amino acids. Tryptophans were determined spectrophotometrically by the method proposed by Beaven and Holiday (1952). Tryptophan content was calculated from molar tyrosine/tryptophan ratios measured spectrophotometically in 0.1 M NaoH, at the wavelengths (280, 294.4 nm).

3.8 Immobilizaton of trypsin

Trypsin was immobilized on Sepharose-4B. Activation of Sepharose–4B was performed as described by Porath *et al.*(1967). For this purpose 10g of Sepharose-4B was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 10.0 ml of distilled water and 10.0 ml of 2 M Na_2CO_3 was added, mixed thoroughly by placing on a magnetic stirrer. Two millilitre of acetonitrile containing 2.0 grams cyanogen bromide was added to the beaker containing Sepharose and the contents were mixed thoroughly at 4^0C for 10 min. The whole mass was immediately transferred to a glass sintered funnel and washed successively with 0.1 M bicarbonate buffer, pH 8.5, distilled water and once again with buffer. After throughly washing the activated Sepharose for 24h in cold. The Sepharose 4B bound protein was separated by centrifugation and protein in supernatant was quantitated in order to calculate the amount of protein immobilized. The protein matrix was thoroughly washed with 0.1 M sodium bicarbonate buffer, pH 8.5 containing 1M sodium chloride and then 0.1 M sodium bicarbonate buffer, pH 8.5. The washed suspension was treated with 0.1ml of 98% ethanolamine for 2h at 4^0C. The matrix was then washed with 0.1 M sodium chloride, distilled water and finally with the buffer.

3.9 Immunological procedures

3.9.1 Immunization of rabbits

Antibodies against buffalo α_2M were raised in healthy male albino rabbits. 250 µg of electrophoretically pure α_2M in 0.5 ml of normal saline emulsified with equal volume of Freund's complete adjuvant was injected subcutaneously. Two weeks later 125 µg of α_2M was injected along with Freund's incomplete adjuvant. A similar second booster dose was given in the third week and the animal was bled after an additional one week. The blood collected was allowed to coagulate at 22°C for 3 h. The antisera was decomplemented at 57°C for 30 min and stored at -20°C in small aliquots.

3.9.2 Immunodiffusion.

Immunodiffusion was performed essentially by the method of Ouchterlony (1962). One percent molten agarose in normal saline containing 0.2% sodium azide was poured in on glass plate and allowed to solidify at room temperature. Required number of wells were cut and the slides were stored at 4^0C. Five to ten microlitres of suitably diluted antisera and required amount of antigen prepared in normal saline were added in different wells. The reaction was allowed to proceed for 12-24 h in a moist chamber at room temperature. The petriplates were immersed in normal saline to remove non-specific precipitin line.

3.10 Dissociation of α_2M and methylamine treated α_2M with NaSCN

Dissociation of α_2M by NaSCN was followed by the method of Shanbhag *et al.* (1996). This was performed by incubating native and methylamine treated α_2M with various concentration of NaSCN in 0.1 M sodium phosphate for 60 min. were then diluted to achieve 0.2 M NaSCN concentration, prior to electrophoresis. The extent of dissociation was determined by electrophoresis using non-denaturing conditions on 5% polyacrylamide gel. Native α_2M incubated with 1%SDS for 90 min at room temperature, was used as marker for the position of the tetrameric and dimeric forms of α_2M respectively. The gel were stained with coomassie brilliant blue R-250.

3.11 Spectroscopy

3.11.1 Fluorescence spectroscopy

Native and zinc treated α_2M subjected to methylamine and trypsin treatment were analyzed by measuring intrinsic fluorescence at 20±0.2°C in Hitachi F 2000 spectrofluorimeter (Tokyo, Japan). The protein was excited (λex) at 280 nm and 295 nm. Corrected emission spectra were recorded with excitation and emission bandwidths of 10 nm. Appropriate controls containing the substances used for the treatment were run and corrections made wherever necessary.

The time course of the change in intrinsic fluorescence at 20±0.2°C was followed by measuring the fluorescence in Hitachi F-2000 spectrofluorimeter. To study tryptophanyl fluorescence α_2M was excited at 295 nm whereas to analyze the cumlative effect of tyrosine, tryptophan and phenylalanine, the protein was excited at 280 nm. Dissociation of native and methylamine-treated α_2M by 1.6 M NaSCN was studied. Solutions of α_2M and NaSCN in 0.1 M sodium phosphate buffer, pH 8.0, were mixed in a fluorescence cuvette to obtain a final concentration of 0.05 mg/ ml of α_2M and 1.6M NaSCN. The temperature was maintained at 22°C by thermostating the cuvette housing during the measurement. The time of mixing was defined as t=0. The fluorescence spectra at known times were recorded and the change in relative fluorescence (ΔF) was calculated at 340 nm.

3.11.2 Circular dichroism spectroscopy

Circular dichroism spectra of native α_2M and that after various treatments were measured in a Jasco J 720 spectropolarimater equipped with a temperature controlled sample cell holder. Spectra were collected with a scan speed of 20 nm/min. and with a response time of 1s. Each spectrum was the average of four scans. Measurements in the far UV (200-250 nm) were taken using α_2M concentrations of about 500 nM (i.e. about 0.35 g/litre), with a 1 mm pathlength cell whereas cells with 10 mm pathlength and α2M concentrations of about 1.5 μM (1.2 g/litre) were used in the near UV (250-300 nm)

region. For each sample, a solution of methylamine or trypsin of the same concentration was used as a blank.

The results were expressed as mean residue ellipticity (MRE) in deg.cm^2.d mol^{-1} which is defined as

$$MRE = \theta_{obs} (m\ deg) / (10 \times n \times l \times c_p) \quad (1)$$

where θ_{obs} is the CD in millidegree, n is the number of amino acid residues, l is the pathlength of the cell and c_p is the mole fraction. The α-helical content of α_2M was calculated from the MRE value at 222 nm using the following equation as described by Chen *et al.* (1972)

$$\% \text{ helix} = [(MRE_{222} - 2340) / 30300] \times 100 \quad (2)$$

The results in the near-UV CD were expressed as molar ellipticiites.

Results

4.0 PURIFICATION AND CHARACTERISATION OF BUFFALO ALPHA-2-MACROGLOBULIN

4.1 PURIFICATION – The purification procedure used in the present study is a combination of ammonium sulphate fractionation and size exclusion chromatography. Buffalo plasma proteins precipitated between 20-40% ammonium sulphate saturation were dissolved in 0.1 M sodium phosphate buffer, pH 8.0 containing 0.1 M KCl, and dialysed against the same buffer. This resulted in 2-fold purification and 80% yield (Table 4.1). Figure 4.1 shows the elution profile of the ammonium sulphate fractionated protein on a Sephacryl S-300 HR column. α_2M was eluted in the first protein peak which was followed by a second peak lacking α_2M activity. The fractions of first peak migrated as a single band in polyacrylamide gel electrophoresis under non-denaturing conditions suggesting homogenity (Fig. 4.2). The two step scheme resulted in 35-fold enrichment in specific activity and a yield of 61% . Purification of buffalo α_2M is summarized in (Table 4.1).

Purification of buffalo α_2M preparation was also followed by PAGE performed both in presence and absence of SDS. Unfractionated plasma and 20–40% ammonium sulphate fractions showed a prominent band of α_2M in addition to several other proteins. After gel filtration the first peak fractions migrated as single band in PAGE (Fig. 4.3). A single band was also obtained when the purified buffalo α_2M was subjected to SDS-PAGE in presence of the thiol reductant β -mercapthoethanol (Fig. 4.4).

4.2 SOME PROPERTIES OF PURIFIED BUFFALO α_2M

4.2.1 Molecular weight - The molecular weight of buffalo α_2M and α_2M half molecule was determined both under native and denaturing conditions. The subunit molecular weight of α_2M was calculated from its mobility in SDS-PAGE by the procedure of Weber and Osborn (1969). The mobilities of marker proteins were plotted against the logarithm of their molecular weights (Fig. 4.5). Least square analysis of the data

TABLE 4.1

Purification of buffalo plasma α_2M

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Plasma	100	6900	638	0.091	1	100
Ammonium sulphate fractionation	25	2940	510	0.173	2.0	80.0
Gel filtration	125	120	390	3.16	35	61.1

Protein concentration was determined by the method of Lowry et al. (1951)

One unit of inhibitory activity is that amount of entrapped trypsin which catalyses the formation of 1 micromole of p-nitroanilide per 30 min.

Figure 4.1 Size exclusion chromatography

The ammonium sulphate precipitate obtained from 100 ml of buffalo plasma was dissolved and dialysed against several changes of 0.1M sodium phosphate buffer, pH 8.0 containing 0.1M KCl at 4^0C. The fraction was applied on Sephacryl S-300 HR column (2.5 x 82cm) and eluted with the buffer at the rate of 30 ml/h. Fractions of 3.0 ml were collected and assayed for α_2M and protein concentration.

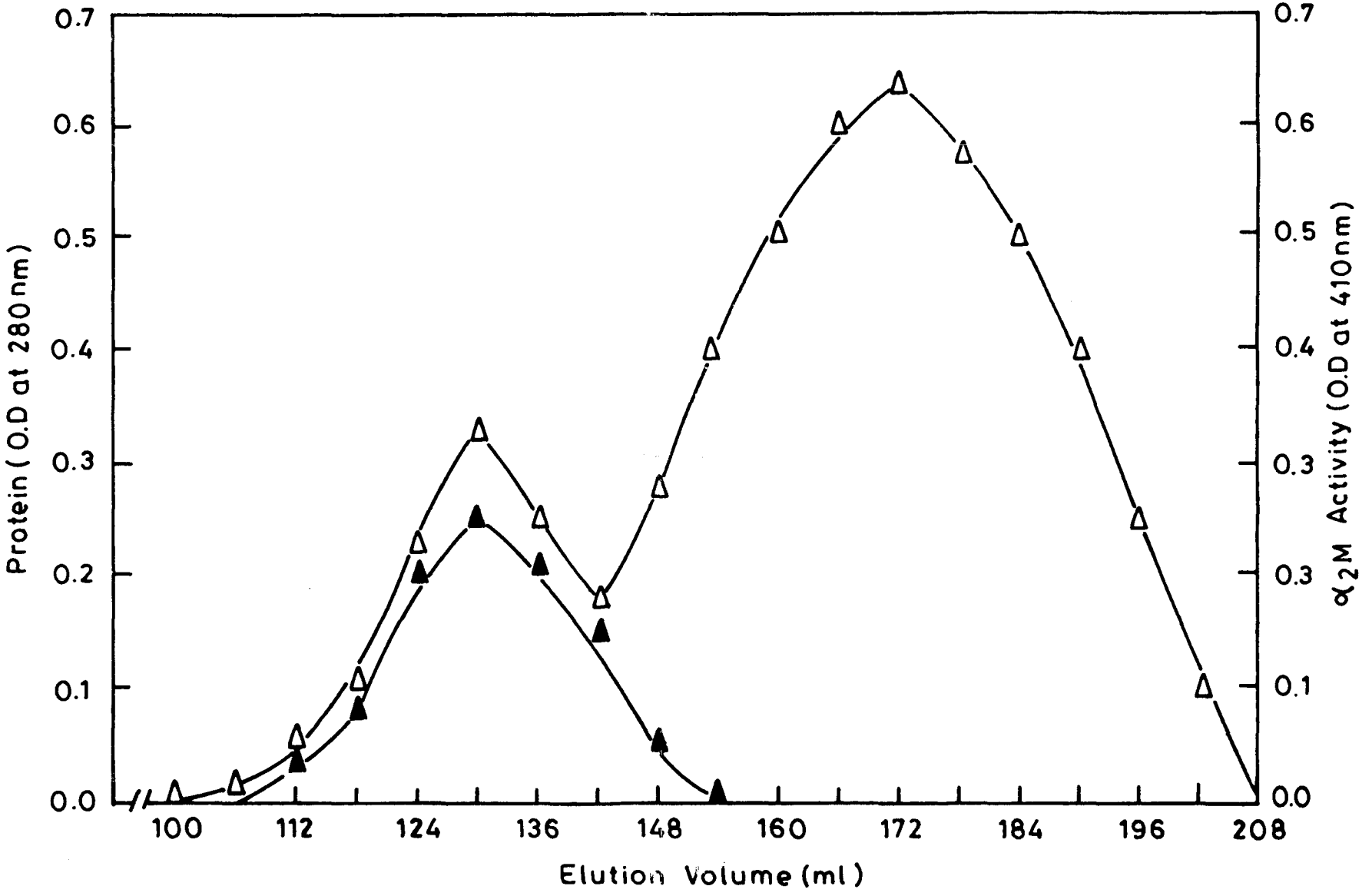
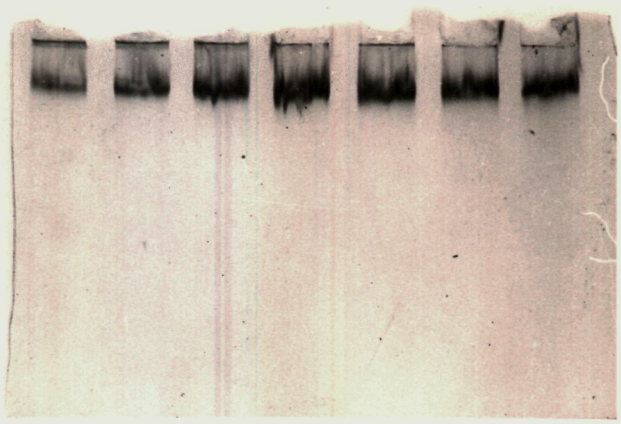

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Protein (O.D at 280 nm)
α_2M Activity (O.D at 410nm)
100
112
124
136
148
160
172
184
196
208
Elution Volume (ml)

Figure 4.2 **Gel electrophoresis of the purified α_2M**

Electrophoresis was performed on 5.0% acrylamide gel as described under materials and methods. Lane a, b, c, d, e, f, g contained fractions 5,7,9,10,12,14 &16 respectively from the first peak of the Sephacryl S-300 HR column. Fifteen μg of protein was applied in each lane.



a b c d e f g

Figure 4.3 **Gel electrophoresis of buffalo α_2M during various stages of purification**

Electrophoresis was performed on 5.0% non-denaturing gel as described under methods. Lane a, b and c contained buffalo plasma, 20-40% ammonium sulphate fraction and the fraction obtained after gel filtration respectively. Fifty μg protein was applied in lanes a and b while lane c contained 15μg.

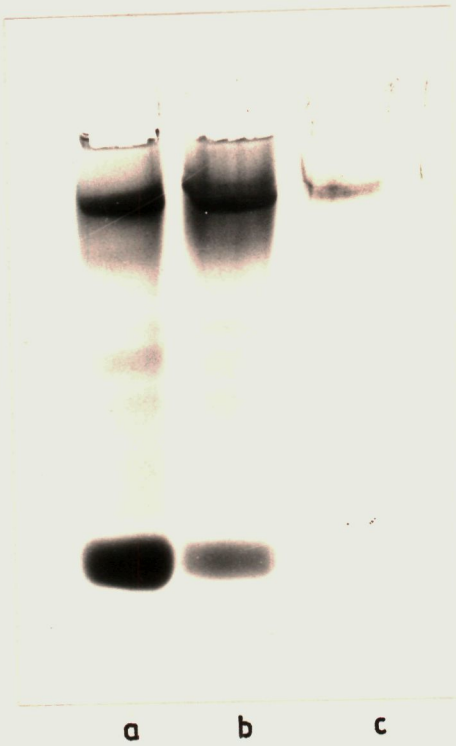

a
b
c

Figure 4.4 **SDS-Polyacrylamide gel electrophoresis at various stages of purification of buffalo α_2M.**

Electrophoresis was performed on 7.5% acrylamide gel as described under methods. Lanes a, b and c contains buffalo plasma, 20-40% ammonium sulphate fraction and the fraction obtained after gel filtration, respectively. 50μg protein was applied in lane a & b and 15μg of protein was applied in lane c.

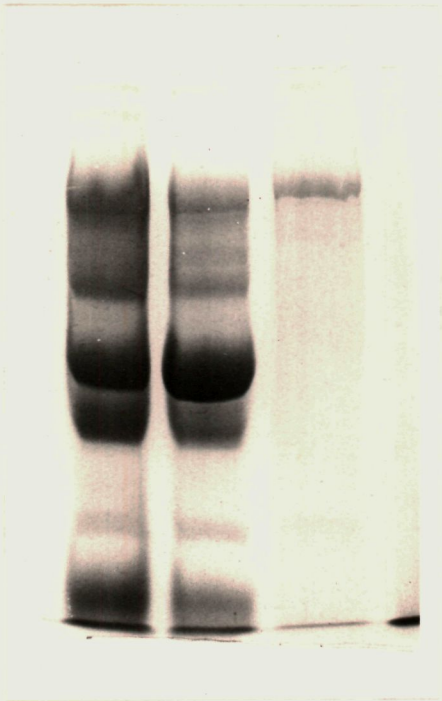
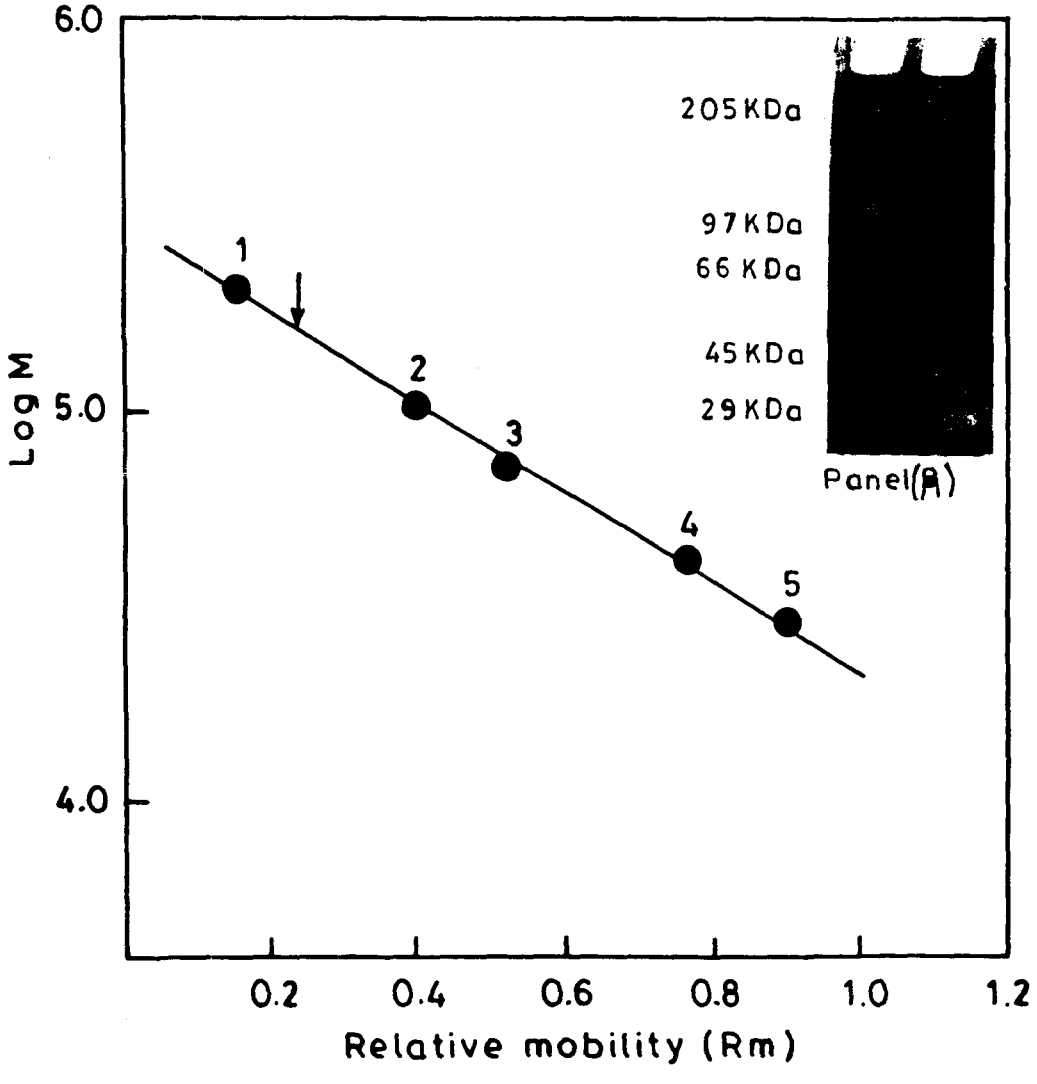

a
b
c

Figure 4.5 **Molecular mass determination of buffalo α_2M by SDS-PAGE**

Electrophoresis was performed on 7.5% acrylamide gel as described in the methods section. The gel lane a contained the molecular mass standards: 1- myosin (205 KDa), 2- phosphorylase b (97.4 KDa), 3- bovine serum albumin (66 KDa), 4- Ovalbumin (45 KDa), 5- carbonic anhydrase (29.1 KDa). Lane b contained 15µg of β-mercaptoethanol treated buffalo α_2M (Panel A). The relative mobility of the marker proteins were plotted against their molecular weight using least square analysis. The position of α_2M subunit is indicated by arrow.


6.0
5.0
4.0
Log M
205KDa
97KDa
66KDa
45KDa
29KDa
Panel(A)
1
2
3
4
5
0.2
0.4
0.6
0.8
1.0
1.2
Relative mobility (Rm)

indicated a linear relationship between log M and relative mobility (Rm) and the position of migration of α_2M subunit corresponded to 165 KDa (Fig. 4.5).

The molecular weight of the native α_2M and α_2M half molecule formed by urea were determined using gel filtration chromatography on Sephacryl S-300 HR. The marker proteins (Thyroglobulin, Apoferritin, β-galactosidase, haemoglobin, and ovalbumin), the purified α_2M and α_2M half molecule were chromatographed on a column of Sephacryl S-300 HR (2.5 X 82 cm) equilibrated with 0.1M sodium phosphate buffer, pH 8.0 and their elution volume determined. Analysis of data indicated a linear relationship between log M and V_e/V_o, where V_e is the elution volume of a protein and V_o is the void volume of the column determined using blue dextran.

The V_e/V_o of native buffalo α_2M and that determined in the presence of 4 M urea corresponded to apparent molecular weight of 660 KDa and 332 KDa respectively (Fig. 4.6).

4.2.2 Stokes Radius - The Stokes radius of buffalo α_2M and urea induced half molecules were determined from their elution volumes from a calibrated Sephacryl S-300 HR column (Fig. 4.7). The column was calibrated by determining the elution volume of several globular proteins with known Stokes radii. The data was analysed according to the theoretical treatment of Laurent and Killander (1964). The linear plot between stokes radii and $[-\log K_{av}]^{1/2}$ of the marker proteins was used for calculating the Stokes radii which were 85^0A and 67.2^0A respectively for the native and half molecules.

4.2.3 Effect of Trypsin - Purified buffalo α_2M treated with two fold molar excess trypsin for 10 min at 37^0C showed the characteristic "slow" to "fast" transformation on electrophoresis in non-denaturing polyacrylamide gels (Fig. 4.8). A similar increase in the electrophoretic mobility was also observed when α_2M was exposed to immobilized trypsin for 2 h at 37^0C (Fig. 4.9).

Figure 4.6 **Molecular weight estimation of native buffalo α_2M and urea induced half molecules using Sepharose-4B gel filtration chromatography.**

Purified buffalo α_2M was applied to a column (2.5 x 82cm) of Sepharose-4B and eluted with 0.1M sodium phosphate buffer (pH 8.0 containing 0.1M KCl) at a flow rate of 30ml/h. The molecular weight markers used were thyroglobulin (TH, 660, KDa), apoferritin (AF, 440 KDa), β-galactosidase (GL, 116 KDa), haemoglobin (Hb, 64 KDa) and ovalbumin (Ova, 45 KDa). The position of elution of native and urea dissociated molecules is indicated with arrows A and B respectively.

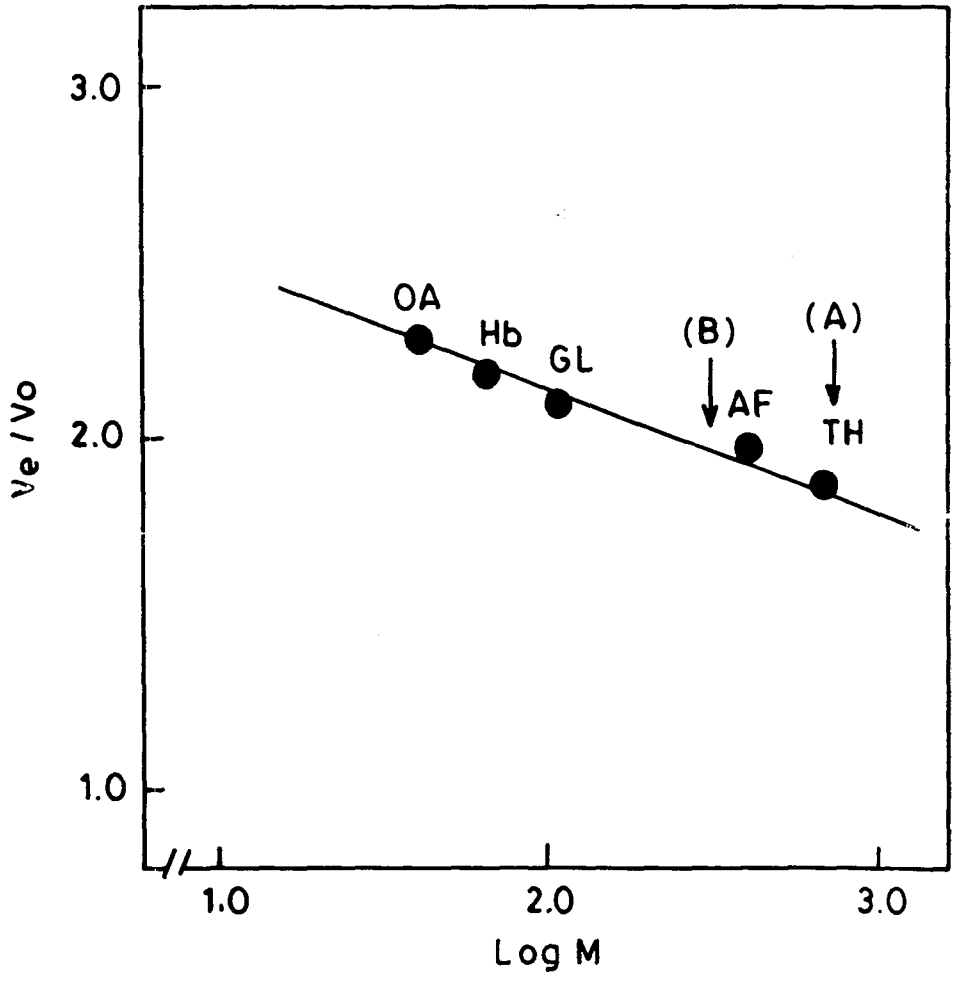

3.0
2.0
1.0
Ve / Vo
OA
Hb
GL
(B)
AF
(A)
TH
1.0
2.0
3.0
Log M

Figure 4.7 **Determination of Stokes radius of buffalo α_2M and α_2M half molecule by Laurent and Killander plot.**

The purified buffalo α_2M, α_2M half molecule obtained by urea treatment and marker proteins were subjected to gel filtration on Sepharose-4B as described earlier. The K_{av} values were computed from the elution volume of marker proteins. Stokes radii for the marker proteins were 1, thyroglobulin (86^0A) 2, appoferritin (59^0A) 3, bovine serum albumin (35.5^0A) 4, ovalbumin (27.3^0A) 5. Soyabean trypsin inhibitor (22.4^0A). The Stokes radii of α_2M, α_2M half molecule formed by urea and NaSCN are indicated by arrows A and B respectively.

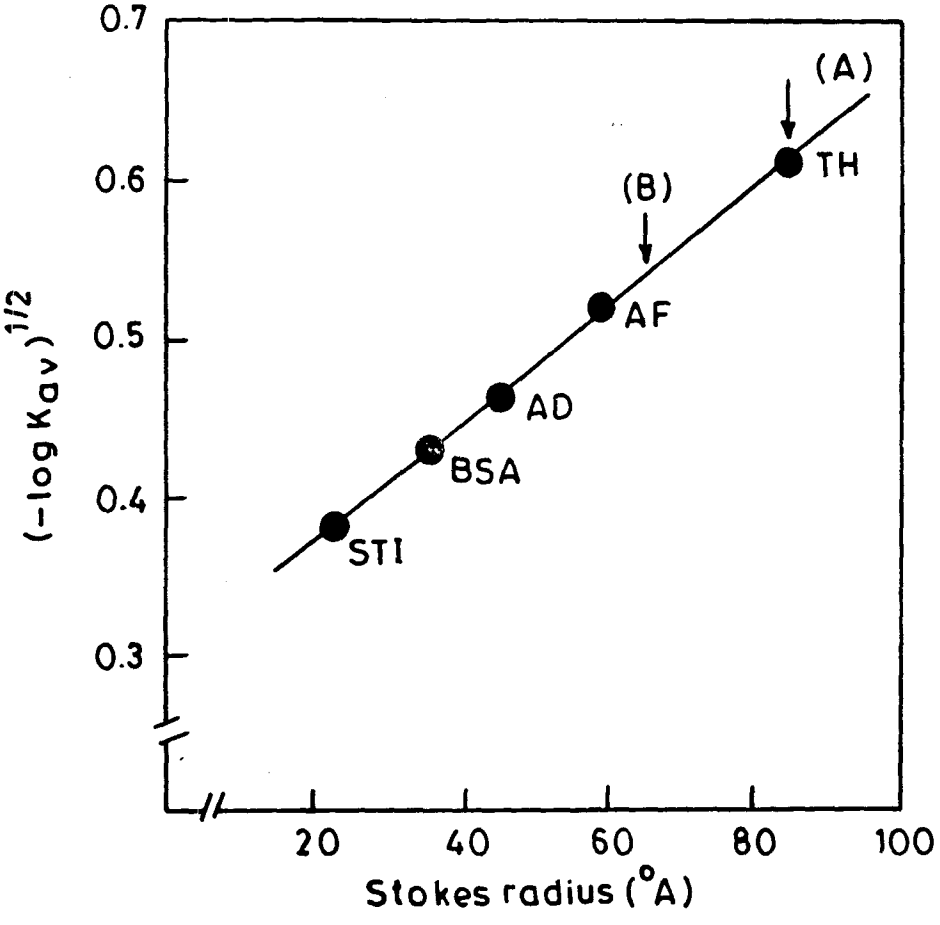

0.7
0.6
0.5
0.4
0.3
(−log Kav)1/2
(A)
TH
(B)
AF
AD
BSA
STI
20
40
60
80
100
Stokes radius (°A)

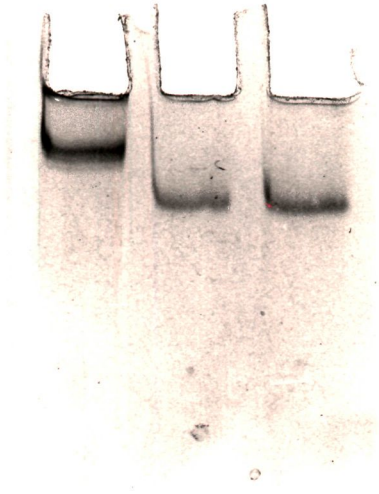
Figure 4.8 **Effect of Trypsinization on buffalo α_2M**

Electrophoresis was performed on 5% non-denaturing acrylamide gel as described under methods. Buffalo α_2M was treated with 3-fold molar excess of trypsin at 37^0C for 10 min and reaction stopped by the addition of 0.001M PMSF. Lane a, native α_2M (20 µg). Lane b, trypsinized α_2M (20 µg).

a b

Figure 4.9 **Gel electrophoresis of buffalo α_2M after treatment with soluble and immobilized trypsin .**

The electrophoresis was performed on 5% non-denaturing acrylamide gel as described in methods section. Buffalo α_2M was treated with 2-fold molar excess of soluble trypsin at 37^0C for 10 min and reaction was terminated by addition of 1mM PMSF and α_2M was treated with 3-fold molar excess of Sepharose-linked trypsin. Lane a, native α_2M. Lane b, α_2M treated with soluble trypsin. Lane c, α_2M treated with immobilized trypsin. 10 µg of protein was applied on each lane.



a b c

4.2.4 Effect of Methylamine - Sensitivity of the thiol ester towards small nucleophiles like methylamine is characteristic of αMs from various sources (Gonias et al.,1982). Buffalo α_2M incubated with 0.5 M methylamine for upto 24h at 22^0C still retained 42% of its original trypsin inhibitory activity as shown in Table 4.2. Incubation of α_2M with methylamine lead only to small increase in electrophoretic mobility that was intermediate between native and trypsin treated α_2M. Trypsinization of the methylamine treated, α_2M however, transformed the intermediate form of the inhibitor to the "fast" form. (Fig. 4.10).

4.2.5 Inhibition of Proteinases – The antiproteinase role of α_2Ms from variety of sources has been identified by inhibition of a broad spectrum proteinases and their protection from inhibition by large molecular weight proteinase inhibitors (Sottrup Jensen, 1989). Buffalo α_2M was also inhibitory towards chymotrypsin (pH 8.0), trypsin (pH 8.0), pronase E (pH 8.0), proteinase K (pH 8.0) and bromelain (pH 6.8). Proteinase inhibition by buffalo α_2M was tested using the high molecular weight substrate casein (Kunitz, 1947). The buffalo α_2M also protected the amidolytic activity of the above enzymes against inhibition by SBTI (Table 4.3).

4.2.6 Reactivity of Thiol esters - Presence of thiol esters is a characteristic feature of the proteins of αM family (Sottrup-Jensen et al., 1980). The number of trypsin or methylamine sensitive thiolester present in α_2M can be determined by the quantitation of free thiols released after the treatments. Native buffalo α_2M contained no free sulphydryls. Trypsinization released 4 mol of thiols per mole of α_2M while those liberated in response to methylamine treatment were only 2 (Table 4.4)

4.2.7 Stoichiometry of Inhibition of Trypsin - The stoichiometry of the association of buffalo α_2-macroglobulin with trypsin was determined by reacting the inhibitor with increasing concentration of trypsin and determining BAPNA-lytic activity of the entrapped trypsin in presence of excess SBTI. As shown in Fig. 4.11, the stoichiometry

TABLE 4.2

Effect of amines on the activity of buffalo α_2M

Treatment	α_2M Activity (units)	Activity retained (%)
None	1.60	100
Methylamine, 10h	0.78	49
Methylamine, 24h	0.664	42
Butylamine, 10h	1.55	96
Butylamine, 24h	1.45	92

Each value represent the mean of three independent experiments performed in triplicate.

TABLE 4.3

Proteinase inhibition by buffalo α_2M

Proteinase	Inhibition	
	Proteolytic inhibitory activity	Amidolytic activity
Bromelain	++	n.d.
Chymotrypsin	++++	Nil.
Pronase E	++	Nil.
Proteinase K	+	Nil.
Trypsin	+++	Nil.

Inhibition of caseinolytic activity was performed as described by Kunitz (1947).

Amidolytic activity of proteinase using BAPNA as substrate in presence of α_2M was determined by Garrot (1966).

TABLE 4.4

Thiol group content of native, trypsin or methylamine treated α_2M

Treatment	Thiol groups (moles/mole α_2M)
None	0.0
Trypsin	4.2 ± 0.08
Methylamine	2.0 ± 0.05
Methylamine followed by trypsin	4.1 ± 0.09

Native, trypsin or methylamine treated α_2M was titrated with DTNB as described under methods and change in absorbance was monitored at 410 nm.

Each value represent the mean of three independent experiments performed in triplicate.

Figure 4.10 Effect of methylamine on buffalo α_2M

PAGE was performed on 5% non-denaturing gel as described under the methods section. Buffalo α_2M was treated with 0.5M methylamine, pH 8.0 for 24h at 22^{0}C as described in the methods. Part of the preparation was treated with 2-fold molar excess of trypsin at 37^{0}C for 10 min and the reaction terminated by the addition of 1mM PMSF. Lane a, contained native α_2M while lane b and c contained methylamine treated and methylamine followed by trypsin treated α_2M. Each lane contained 15-20 µg of protein respectively .

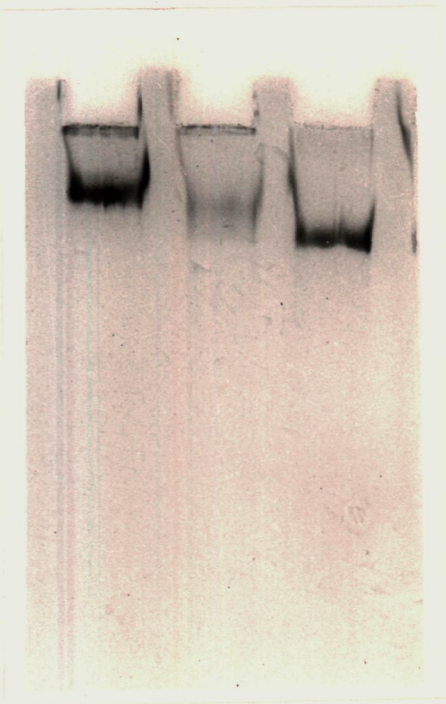
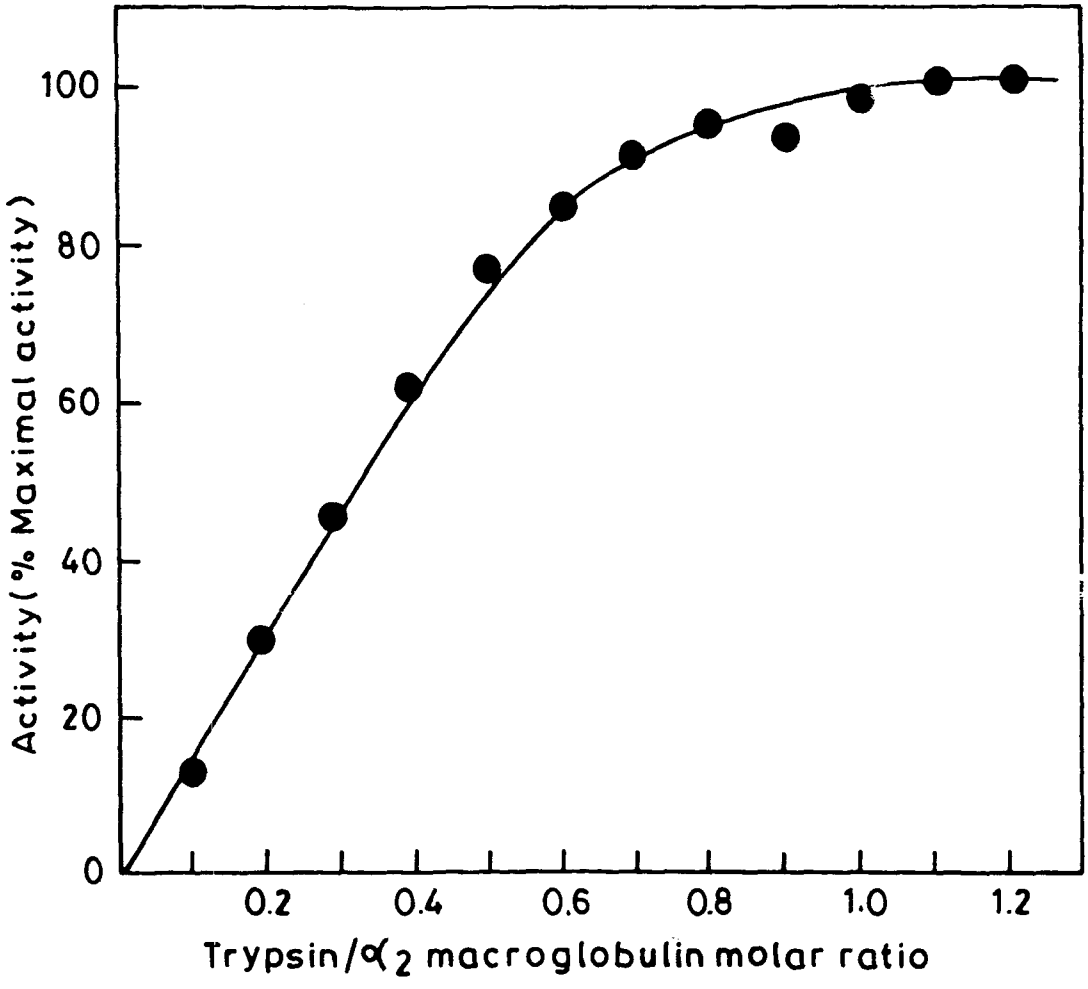

a
b
c

Figure 4.11 Binding site Titration of buffalo α_2M

Buffalo α_2M (0.18 nmoles) was incubated with various concentrations of trypsin in 10mM sodium phosphate buffer, pH 8.0 at 37^0C for 15 min. This was followed by addition of 0.45 nmoles of SBTI and incubation at 37^0C for another 15 min. The amount of trypsin protected from SBTI by α_2M was assayed by the addition of BAPNA as described in the methods. The percentage of maximal activity is defined as the ratio of esterase activity for a given trypsin : α_2M ratio divided by the highest esterase activity found for trypsin α_2M in this study (Hudson et al., 1987).


100
80
60
40
20
0
Activity (% Maximal activity)
0.2
0.4
0.6
0.8
1.0
1.2
Trypsin/α2 macroglobulin molar ratio

of inhibition of trypsin showed that each mole of intact buffalo α_2M entrapped 1.0 mol of the enzyme. The value was thus lower than that of the human inhibitor which can entrap upto 2.0 mol of proteinase per mole of α_2M.

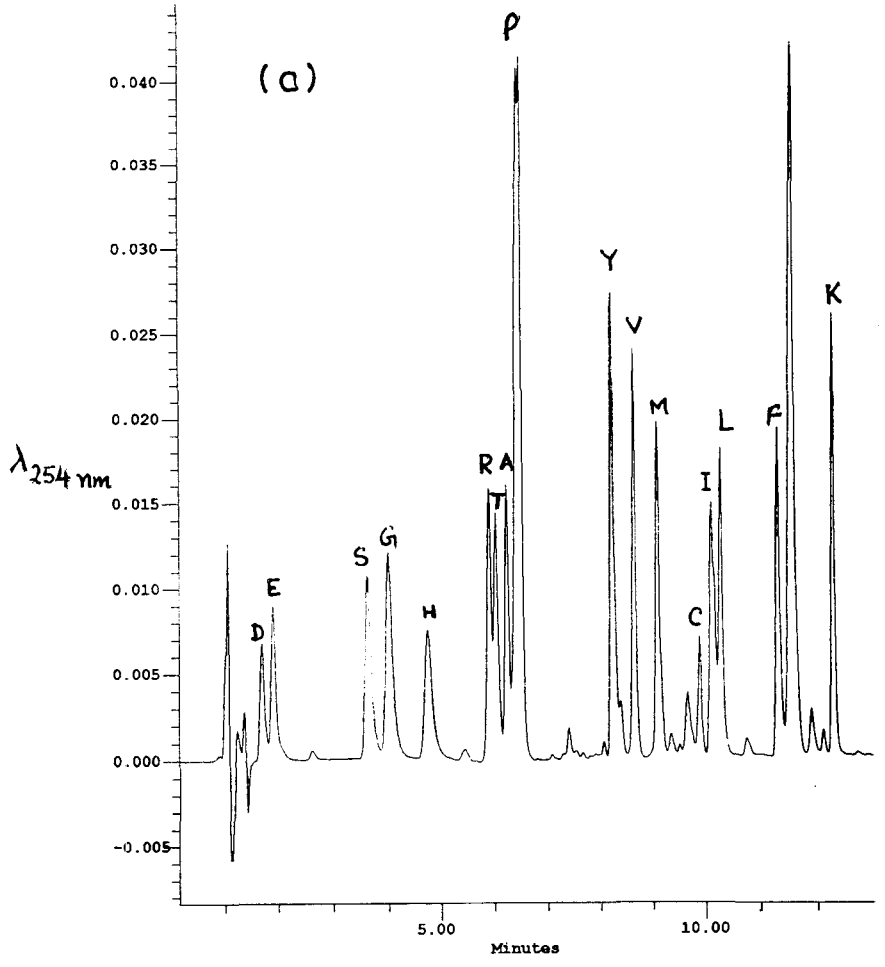
4.2.8 Carbohydrate Composition - α_2Ms from a variety of sources are glycosylated to various extents and buffalo α_2M was no exception with a total hexose content of 7.8%. This value is lower than the human α_2M, that contains 9.43% carbohydrate (Dunn and Spiro, 1967).

4.2.9 Amino acid composition – The concentration of each amino acid was estimated by measuring the area of the respective peak by matching it with the standard amino acid peak (Fig 4.12). The data for the amino acid composition of buffalo α_2M (Table 4.5) shows good similarities, with the α_2M's of goat, mouse and human plasma. Concentration of each amino acid was estimated by measuring the area of the respective peak by matching with the standard amino acid peak (Fig. 4.12 a & b). The buffalo α_2M is however significantly deficient in proline and aspartic acid, but rich in alanine as compared to goat, mouse and human inhibitors.

4.2.10 Cross reactivity - Buffalo α_2M was quite immunogenic and readily induced antibody formation in rabbits. Fig 4.13 shows that antisera raised against purified buffalo α_2M readily cross reacted with goat and human α_2M as indicated by the formation of precipitin lines.

Figure 4.12 (a) Standard Amino acid spectra.

(b) Amino acid composition spectra of buffalo α_2M.


(a)
λ254 nm
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
D
E
S
G
H
R
T
A
P
Y
V
M
C
I
L
F
K
5.00
10.00
Minutes

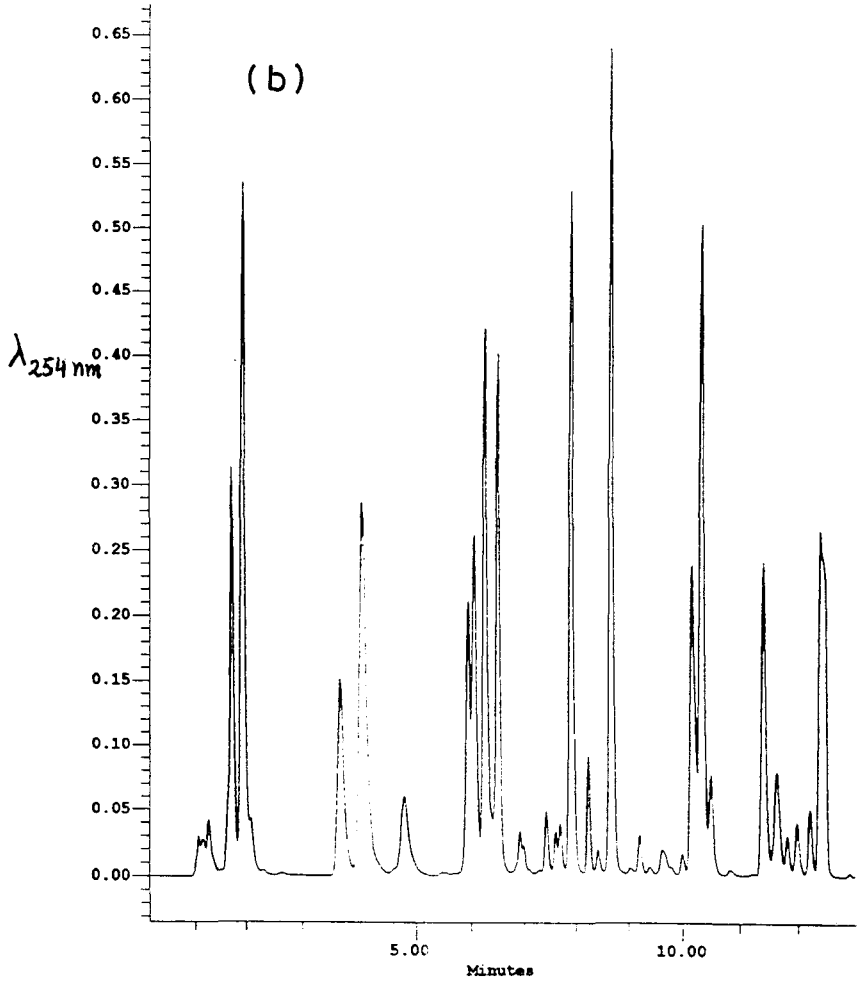

(b)
λ254 nm
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5.00
10.00
Minutes

TABLE 4.5

Amino acid composition of the purified buffalo, goat, mouse and human α_2M

Amino acid	Composition (mol/100 mol of residues)			
	Buffalo α_2M[a]	Goat α_2M[b]	Mouse α_2M[c]	Human α_2M[d]
Asp	6.92	8.18	9.56	7.95
Thr	5.32	7.59	6.28	6.75
Ser	8.89	10.58	7.97	8.35
Glu	11.49	11.50	11.54	12.25
Pro	3.88	6.07	4.87	5.25
Gly	7.48	6.87	5.90	6.20
Ala	8.35	6.04	5.62	6.35
Cys	1.20	2.07	1.22	1.61
Val	9.04	8.56	8.34	9.45
Met	1.63	1.13	1.53	1.72
Ile	4.69	3.62	5.44	3.93
Leu	9.80	8.42	9.65	9.25
Tyr	3.90	3.60	3.19	3.86
Phe	4.17	3.56	4.20	4.27
Lys	6.57	5.95	5.85	6.14
His	2.86	1.95	4.87	2.69
Arg	3.90	3.60	3.00	3.86
Trp*	1.13	0.58	1.03	0.76

The results are based on Mr value of 660,000 for buffalo α_2M.

Sample [1mg] was hydrolysed for 24 hrs at 110^{0}C under vacuum in 6 N HCl predistilled with constant boiling. Amino acid compositional analysis was carried out on Beckman 121 M analyzer

- Trp was calculated by the method of Beaven and Holiday (1952)

a. This study

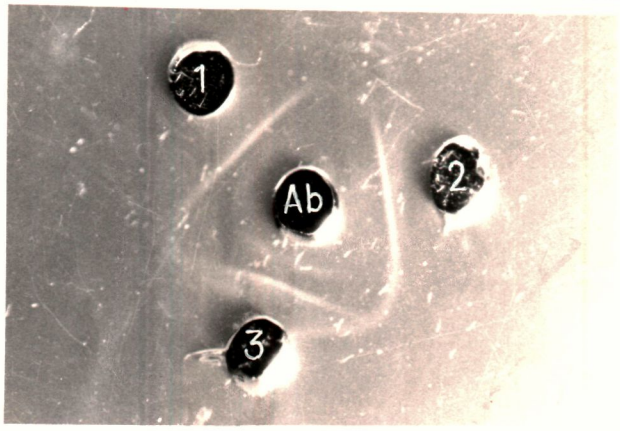
b. Unpublished data of Dr. Fahim. H. Khan (1996)

c. Hudson *et al.* (1987)

d. Dunn and Spiro (1967)

Figure 4.13 Ouchterlony Immunodiffusion

Antibuffalo α_2M antisera were raised in albino rabbits. The immunodiffusion of antibuffalo α_2M antisera was allowed to react with buffalo and human α_2M in agarose gel as described in the methods section. The central well contained antibuffalo α_2M antisera and well 1, 2 and 3 contained buffalo, goat and human α_2M respectively.


1
Ab
2
3

4.3 DISSOCIATION OF NATIVE AND MODIFIED ALPHA-2-MACROGLOBULIN BY SODIUM THIOCYANATE (NaSCN)

4.3.1. Effect of NaSCN on α_2M

Sodium thiocyanate is a chaotropic agent which causes the disruption of hydrophobic forces. When native α_2M was incubated for 60 min with increasing concentration of NaSCN, the process of dissociation to dimers became significant once the concentration of the salt equals 1.2 M. The proportion of α_2M half molecules increases with further increase in the concentration and at 2.0 M NaSCN most of the inhibitor was in the form of dimers (Fig. 4.14). The dissociation into dimers was not readily reversible as relowering of NaSCN the concentration to 0.2 M resulted in no major increase in the fraction of tetrameric molecule.

4.3.2. Effect of NaSCN on methylamine modified α_2M

α_2M treated with methylamine (α_2M-$MeNH_2$) was more resistance to dissociation by NaSCN. Significant dissociation of α_2M-$MeNH_2$ was apparent only at 1.4 M NaSCN and even at higher concentration of NaSCN the proportion of the half molecules formed from α_2M-$MeNH_2$ was lower than that obtained from the native α_2M (Fig. 4.15).

4.3.3. Stokes radius

Native α_2M has a Stokes radius of 85°A. The elution profile of the α_2M half molecules obtained by dissociation of α_2M with 1.6 M NaSCN gave a Stokes radius (R_e) of 65.2 °A. The Re of the half molecules obtained by dissociation of α_2M by NaSCN was comparable with that obtained by dissociation of α_2M with 4M urea (R_e = 67.2°A) as given in table 4.6.

4.3.4. The effect of NaSCN on the intrinsic fluorescence of α_2M

Intrinsic fluorescence of protein is contributed by three fluors –tryptophans,

Figure 4.14. Gel electrophoretic analysis of the dissociation of buffalo α_2M by sodium thiocyanate.

Electrophoresis was performed on 5% non-denaturing gel as described under methods section. The effect of incubation with different concentrations of NaSCN for 60 min at 22°C. The salt concentrations are in lanes : a, zero, b, 1.0 M; C, 1.2 M; d, 1.4; e, 1.6 M; and f. 2.0 M, Lane g contains half-molecules obtained by incubation of α_2M with 1% SDS for 90 min at 22°C. All lanes contain 25 μg protein.

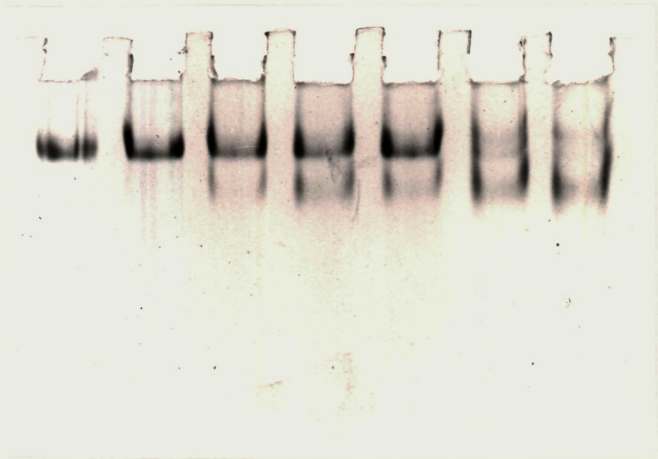

a b c d e f g

Figure 4.15. **Polyacrylamide Gel electrophoresis of the dissociation of methylamine modified α_2M by sodium thiocyanate.**

Electrophoresis was performed on 5% acrylamide gel as described under methods. The effect of incubation of α_2M-$MeNH_2$ with different concentrations of NaSCN for 60 min at 22°C. Lanes: a, α_2M; b, α_2M-methylamine. The salt concentrations in lanes are : c, 1.2 M; d, 1.4 M; e, 1.6 M and f, 2.0 M. Lane g, contains half molecules obtained by incubation of α_2M with 1% SDS for 90 min at 22°C. All lanes contain 25 µg protein

a b c d e f g

TABLE 4.6

The stoke's radii, (Re), of buffalo α_2M and α_2M half molecule

Treatment	Re (^{0}A)
None	85.0
4M urea	67.2

tyrosine and phenylalanine. Excitation at 295 nm results primarily in tryptophan fluorescence, but with minimal interference from tyrosine, whereas excitation at 280 nm is the cumulative result of tryptophan, tyrosine and phenylalanine fluorescence.

When native α_2M and α_2M-$MeNH_2$ were incubated for 80 min. with 1.6 M NaSCN and excited at 280 and 295 nm (Fig. 4.16 & 4.17) the relative fluorescence decreased. The observed small red shift in the emission maximum for native and methylamine modified α_2M after 80 min. probably indicates that the environment of the tryptophan, tyrosine and phenylalanine involved is slightly less hydrobhobic in α_2M half molecules than in α_2M.

The curves in Fig. 4.18 and 4.19 depict the rates of dissociation of native and methylamine treated α_2M induced by incubation with 1.6 M NaSCN. The rate of change in relative fluorescence at 340 nm when λ_{ex} = 295 and 280 nm, was measured. These dissociation were $7.46 \pm 0.26 \times 10^{-4}$ s^{-1} and $3.26 \pm 0.07 \times 10^{-4}$ s^{-1} for native α_2M and α_2M-$MeNH_2$ at λ_{ex} = 295 nm respectively. The curve for native α_2M and α_2M-$MeNH_2$ in Fig. 4.19 shows that 90% of the final change in fluorescence (ΔF max) was reached in 50 min at 295 nm. The fluorescence changes at 280 nm was slower and required nearly 80 min for completion (Fig. 4.18). The first order rate constant for native α_2M and α_2M-$MeNH_2$ at λ_{ex} = 280 nm as calculated from the data in Fig. 4.18 is $9.48 \pm 0.06 \times 10^{-4}$ s^{-1} and $4.5 \pm 0.25 \times 10^{-4}$ s^{-1} respectively. The k (rate constant) for native α_2M is thus almost 2 times higher for α_2M than α_2M-$MeNH_2$ when excited either at 295 or 280 nm.

Figure 4.16. Intrinsic fluorescence of α_2M and α_2M-$MeNH_2$ on incubation with sodium thiocyanate.

The intrinsic fluorescence of α_2M and α_2M-$MeNH_2$ on incubation with 1.6 M NaSCN at 22°C. The wavelength of excitation, (λex), was 280 nm and the emission spectra between 320 and 360 nm were collected at zero time and after 80 min, with the time of mixing the solutions of α_2M and NaSCN taken as zero. The concentration of α_2M and α_2M-methylamine in the curette was 0.2 mg/ml. The slit width was 10 nm for the excitation and emission beam, respectively. The spectra for the following are shown by

(—) α_2M at zero time

(.....)α_2M after 80 min

(– · –) α_2M- $MeNH_2$ at zero time

(-----) α_2M- $MeNH_2$ after 80 min.

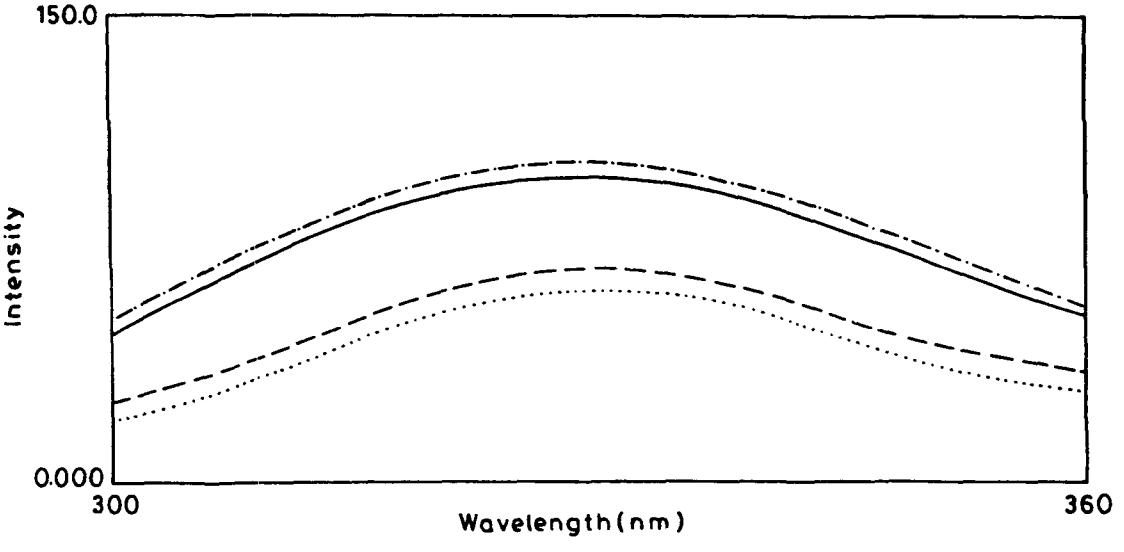

150.0
0.000
Intensity
300
360
Wavelength(nm)

Figure 4.17. Tryptophanyl fluorescence of α_2M and α_2M- $MeNH_2$ on treatment with sodium thiocyanate.

The intrinsic fluorescence of α_2M and α_2M- $MeNH_2$ with 1.6 M NaSCN at 22°C. The wavelength of excitation, (λex), was at 295 nm and the emission spectra between 320 and 360 nm were collected at zero time and after 80 min, with the time of mixing the α_2M and NaSCN; α_2M- $MeNH_2$ in the cuvette was 0.2 mg/ml. The slit width was 10 nm for the excitation and emission beam, respectively.

(—) α_2M at zero time

(.....)α_2M after 80 min

(– · –) α_2M- $MeNH_2$ at zero time

(-----) α_2M- $MeNH_2$ after 80 min.

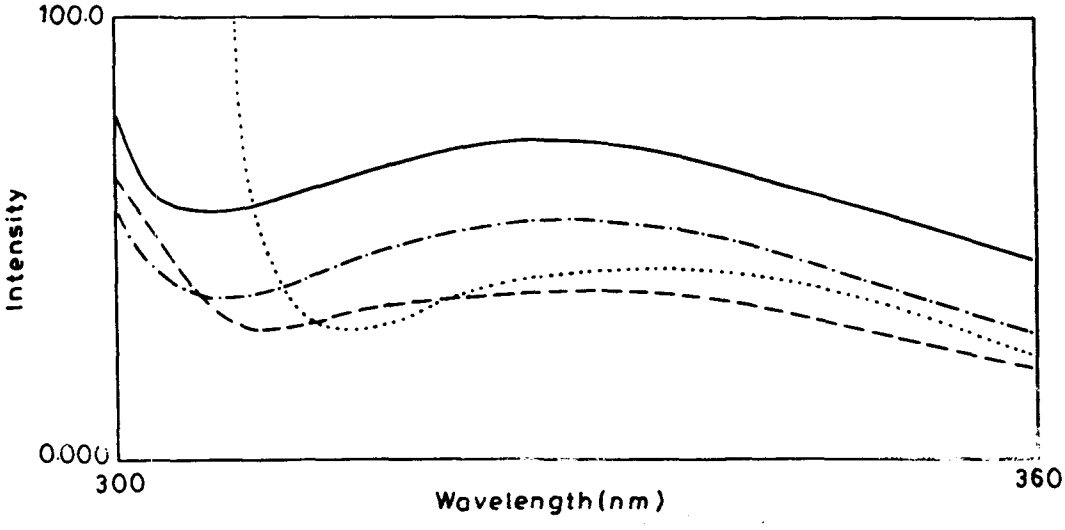

100.0
0.000
Intensity
300
360
Wavelength (nm)

Figure 4.18 The rate of change in the relative fluorescence (ΔF) of α_2M and α_2M-$MeNH_2$ at 340 nm on incubation with 1.6 M NaSCN at 22^{O}C at 280 nm.

(○) α_2M

(●) α_2M-$MeNH_2$

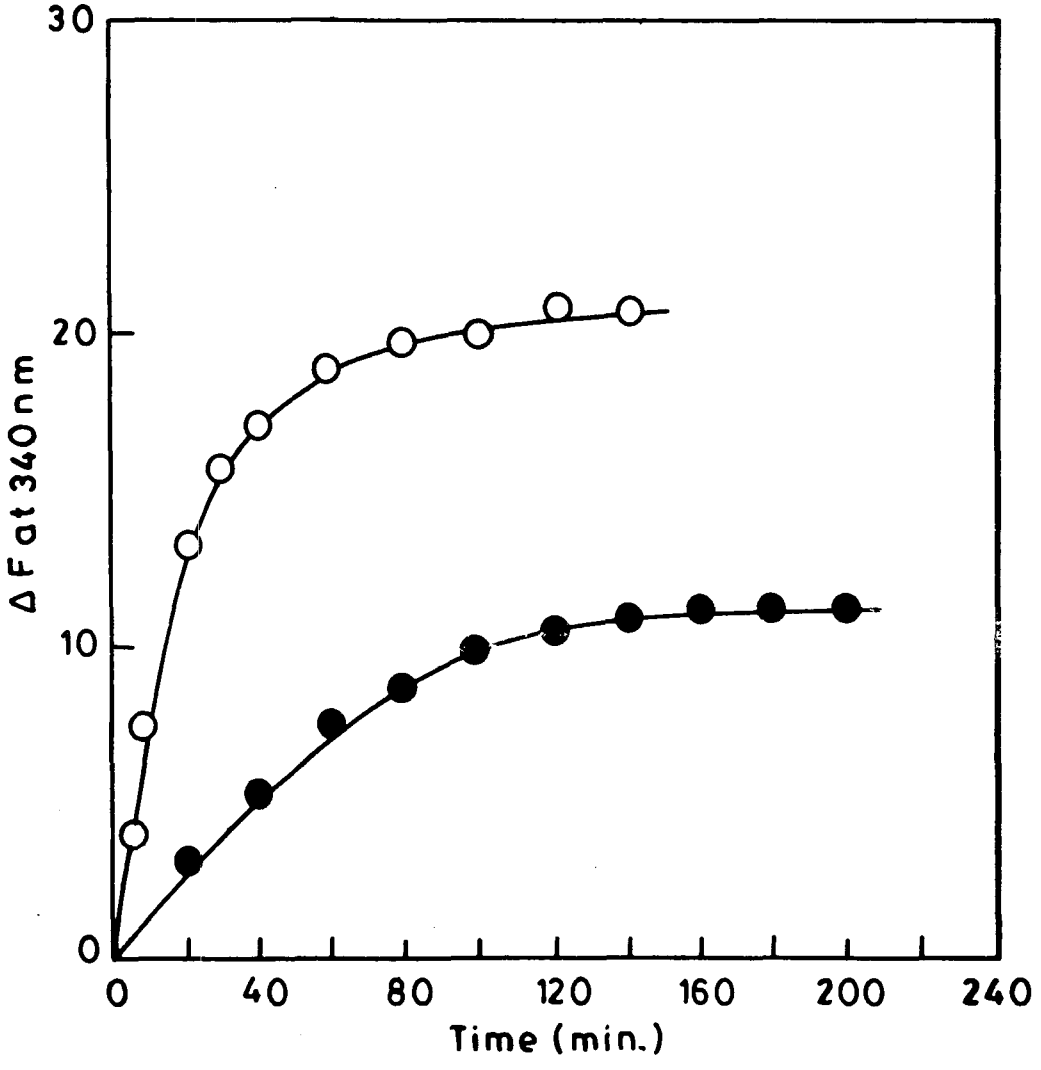
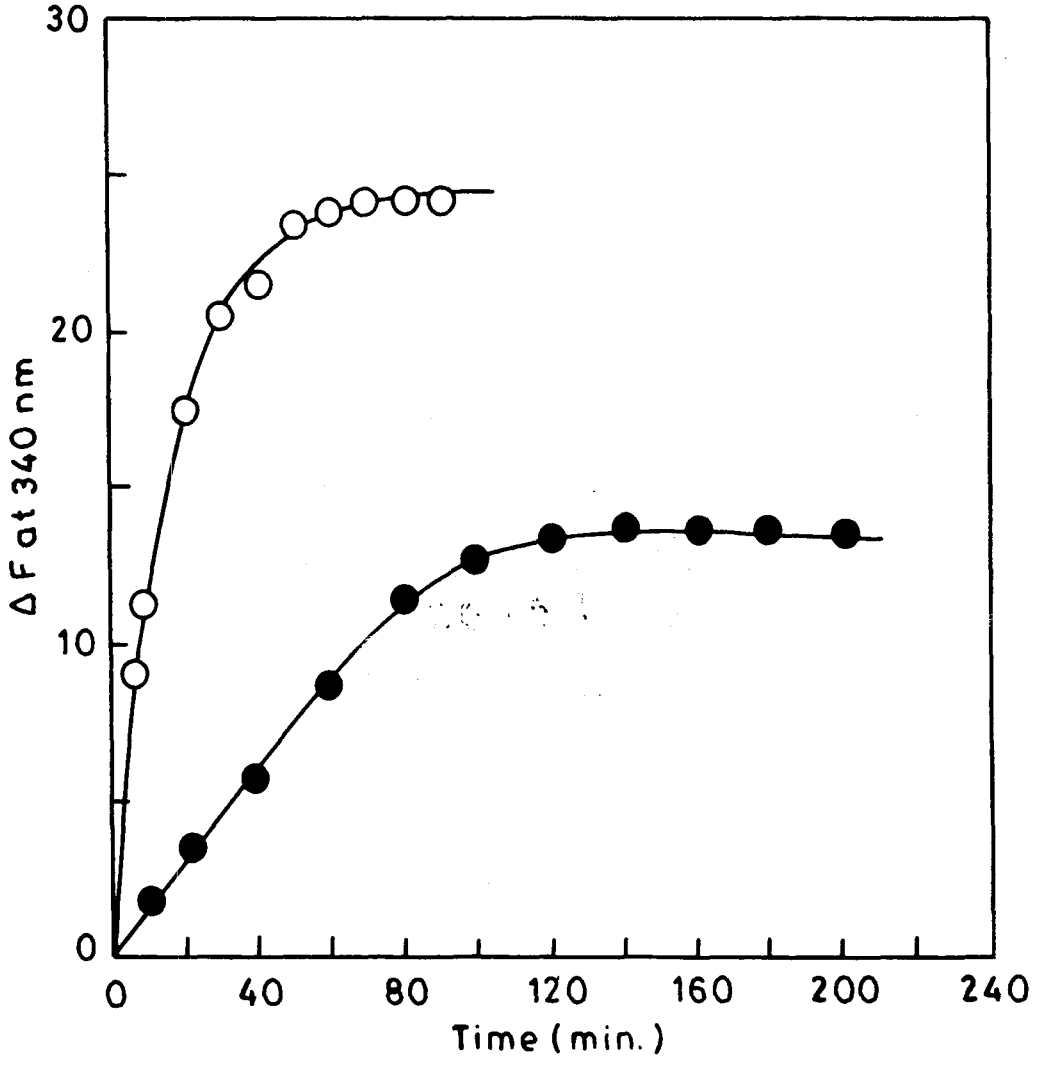

30
20
10
0
ΔF at 340nm
0
40
80
120
160
200
240
Time (min.)

Figure 4.19. The rate of change in relative fluorescence (ΔF) of α_2M and α_2M-$MeNH_2$ at 340 nm on incubation with 1.6 M NaSCN at 22°C The data at 295 nm.

(○) α_2M

(●) α_2M-$MeNH_2$


30
20
10
0
ΔF at 340 nm
0
40
80
120
160
200
240
Time (min.)

4.4. EFFECT OF ZINC ON STRUCTURE AND FUNCTION OF α_2M

4.4.1. Plasma and α_2M zinc concentration

The physiological zinc concentration of buffalo plasma determined by the method of Song et al. (1976) was in the range of 18 – 20 μM. The purified buffalo α_2M contained 400 μg Zn/g (6g-atom zinc) of protein. Irreversible binding of zinc by the α_2M was also estimated after treatment with 30 μM and 200 μM zinc for 1 h at 4^0 C. Prior to zinc analysis this preparation was extensively dialysed against 20 mM Tris buffer, pH 8.0 in order to remove the loosely bound metal zinc. The zinc content of the sample incubated with 30 μM and 200 μM zinc was 1,055 μg and 3,800 μg/g of protein, respectively.

4.4.2. The Effect of zinc on trypsin binding activity of α_2M

The effect of zinc on trypsin binding activity of the buffalo α_2M is shown in Fig. 4.20 the trypsin binding activity of α_2M does not depend on the presence of zinc since all but traces of this metal can be removed by EDTA without significant loss of the trypsin binding. Incubation with higher concentration of zinc however interfered with trypsin binding activity. At 30 g-atom Zn/g, over 50% of trypsin binding activity was lost and when the concentration was 58g-atom Zn per/g α_2M practically all binding activity disappeared. Exhaustive dialysis of α_2M samples incubated with higher concentrations of zinc against 20 mM Tris buffer, pH 8.0 containing EDTA did not restore its trypsin binding activity.

4.4.3. EFFECT OF ZINC ON THE CONFORMATION OF α_2M

4.4.3.1. Gel Electrophoresis

Trypsinization of the native buffalo α_2M resulted in the characteristics "slow" to "fast" transformation in electrophoretic mobility. The inhibitor pre-treated either with 30 μM or 200 μM zinc also exhibited the transformation (Fig. 4.21). Incubation with zinc at either concentration also did not alter the response of the buffalo α_2M methylamine treatment. The treatment resulted only in small increase in electrophoretic mobility (Fig. 4.22).

Figure 4.20. Effect of zinc on the trypsin binding activity of α_2M.

To six of a series of 9 tubes each containing (2 mg/ml with a zinc content of 5 g-atoms Zn/mol), solution of $ZnCl_2$ was added to make a final concentration of about 10, 30, 100, 200 and 500 μM and the trypsin binding activity measured. The remaining 3 tubes were dialyzed for 48 h against 1, 5 and 10 mM EDTA respectively in 20 mM Tris buffer, pH 8.0 and the zinc content of α_2M as well as trypsin binding activity determined. The bar in the graph represent g-atoms Zn/mol of α_2M

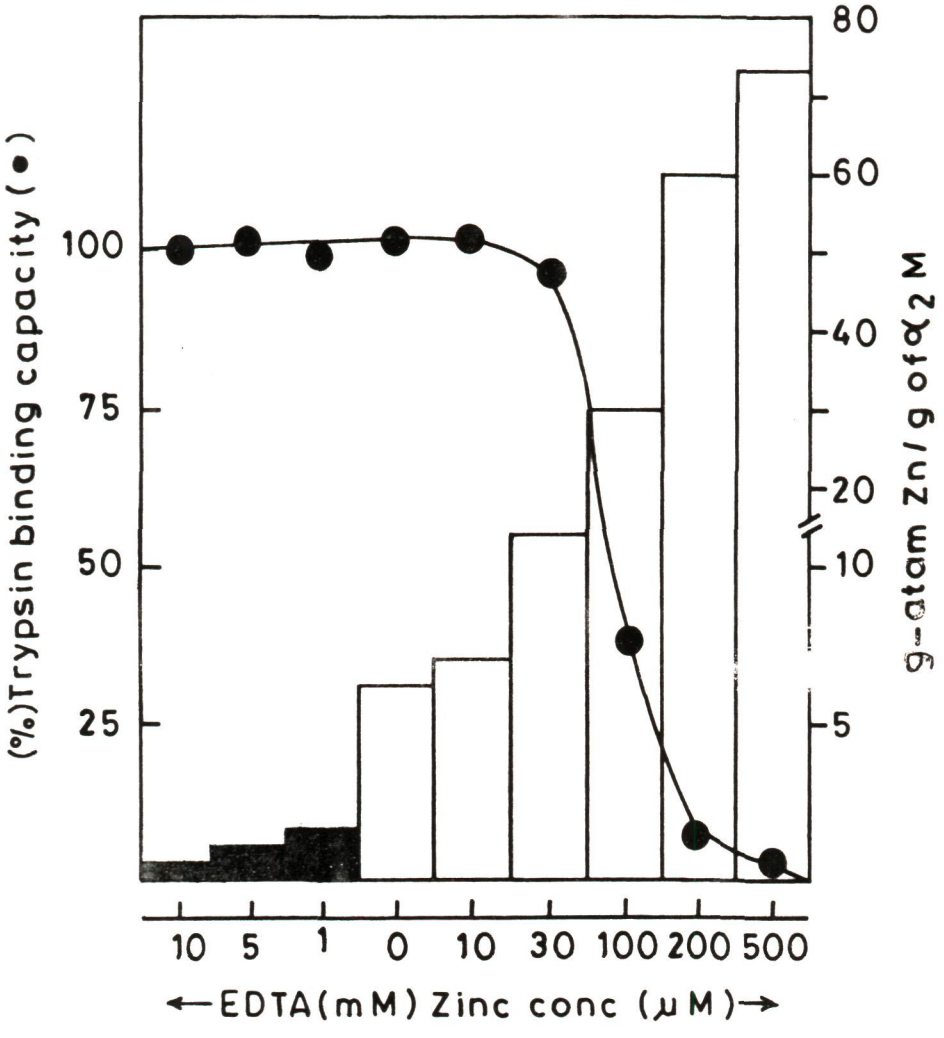

(%)Trypsin binding capacity (•)
100
75
50
25
80
60
40
20
10
5
g-atam Zn/g of α2 M
10 5 1 0 10 30 100 200 500
←EDTA(mM) Zinc conc (μM)→

Figure 4.21. Effect of trypsin on native and zinc treated α_2M.

Electrophoresis was performed on 5% acrylamide gel as described under method section. Buffalo α_2M treated with zinc was reacted with 3-fold molar excess of trypsin at 37°C for 10 min and reaction was stopped by addition of 1mM PMSF. Lane a, c, and e contains native, 30 μM and 200 μM zinc treated α_2M respectively. Lane b, d, f contains there trypsinized forms respectively. All lanes contain 20 μg protein.

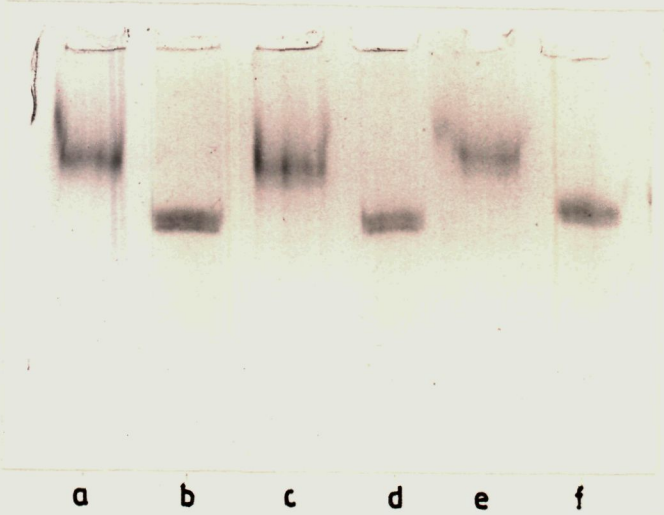
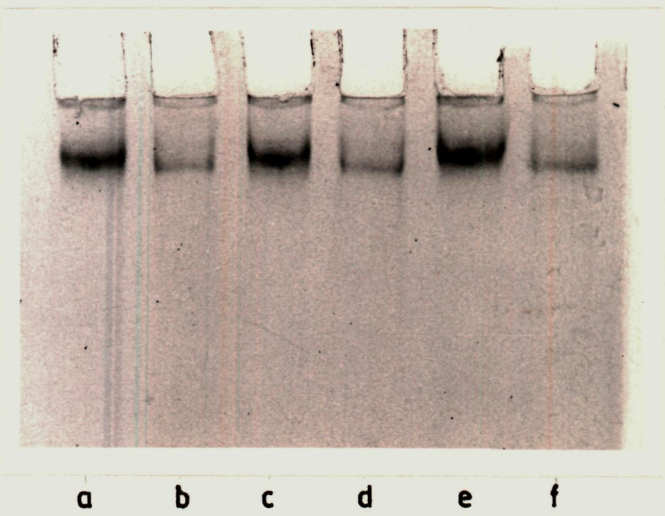

a
b
c
d
e
f

Figure 4.22. Effect of methylamine on native and zinc treated α_2M.

PAGE was performed on 5% gel as described in the methods section. Buffalo α_2M and zinc reacted α_2M was treated with 0.5 M methylamine, pH 8.0 for 24 h at 22^0C as described in text. Lane a, contained native α_2M; Lane c, α_2M treated with 30 μM zinc; Lane e, 200 μM zinc treated α_2M; Lane b, methylamine treated form of native α_2M; Lane d and f contains methylamine reacted form of 30 μM and 200 μM zinc treated forms respectively. 20 μg of protein was applied in each lane.


a
b
c
d
e
f

4.4.3.2. Intrinsic Fluorescence

The corrected fluorescence emission spectra obtained for buffalo α_2M alone gives emission maximum at 333nm (Fig. 4.23) when excited at 280nm. Reaction with methylamine caused a 3nm blue shift of the emission maximum and was accompanied by a small enhancement in the fluorescence. Treatment with Sepharose-linked trypsin resulted in 2nm red shift with 50% decrease in the magnitude of fluorescence.

To observe tryptophanyl fluorescence, native α_2M was excited at 295nm. This resulted in an emission maximum at 339nm. As compared to native α_2M, methylamine treated α_2M showed a 3nm blue shift with no significant change in magnitude of fluorescence. Native inhibitor on reaction with immobilized trypsin responded with a 2nm blue shift but with 28% decrease in fluorescence as shown in Fig 4.24.

In order to study the effect of zinc on the methylamine and trypsin induced alteration in the tertiary structure the modified α_2M was excited at 280nm. α_2M treated with 30μM zinc retained nearly 80-100% activity showed conformational alterations comparable with those of native α_2M (Fig. 4.25). Treatment with 200 μM zinc however, resulted in inactivation of α_2M and the α_2M pretreated thus exhibited 30% quenching indicating specific conformational changes in the molecule on binding of zinc. Treatment with methylamine resulted in 18-20% enhancement in fluorescence with a 3nm blue shift in emission maxima whereas 3nm red shift was observed with Sepharose-linked trypsin with 25% quenching (Fig. 4.26).

4.4.3.3. Circular dichroism of native and zinc treated alpha-2-macroglobulin

4.4.3.3.1. Circular dichroism spectra in far-U.V. region

The spectra for native α_2M is characterized by the presence of a minima at 216 nm with a shoulder around 213 nm. Reaction of the inhibitor with methylamine resulted only in marginal alteration in the spectrum while, Sepharose-linked trypsin caused very marked decrease in the mean residue elllipticity (Fig. 4.27).

Figure 4.23. Intrinsic fluorescence of α_2M and α_2M treated with methylamine and Sepharose linked trypsin.

Corrected fluorescence spectra of buffalo α_2M alone and α_2M reacted with methylamine and Sepharose linked trypsin (——) α_2M alone; (— · —) α_2M reacted with methylamine; (------) α_2M reacted with Sepharose-linked trypsin. The conditions of the reactions are given in methods section. The excitation wavelength was 280 nm, and bandwidth was 10 nm in both channels. The α_2M concentration was 0.18 μM.

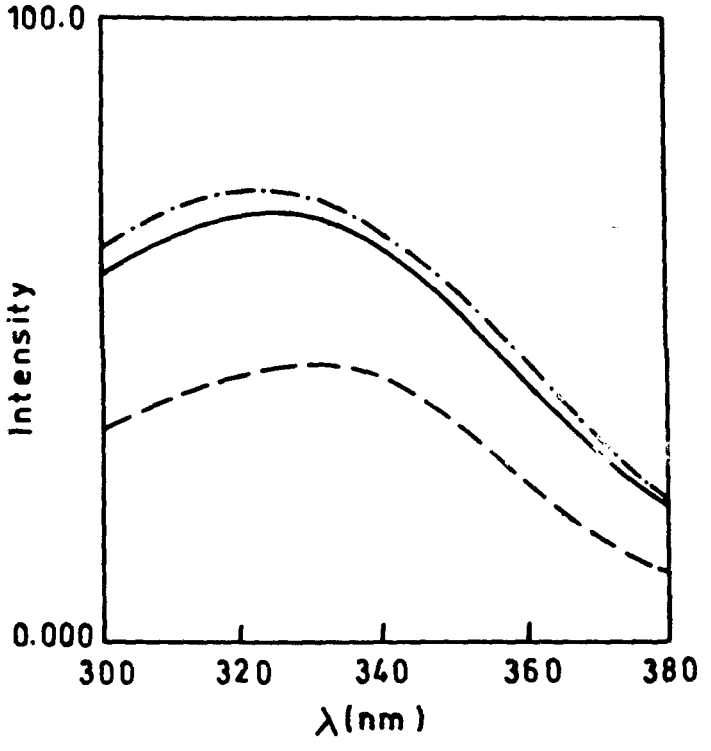

100.0
0.000
Intensity
300
320
340
360
380
λ(nm)

Figure 4.24. Intrinsic fluorescence of native α_2M and α_2M reacted with methylamine and immobilized trypsin at 295 nm.

Corrected fluorescence spectra of α_2M alone and α_2M reacted with methylamine and Sepharose-linked trypsin. (——) α_2M alone; (— · —) α_2M reacted with methylamine; (------) α_2M reacted with immobilized trypsin. The conditions of the reactions are given in methods section. The excitation wavelength was 295 nm, and bandwidth was 10 nm in both channels. The α_2M concentration was 0.18 μM.

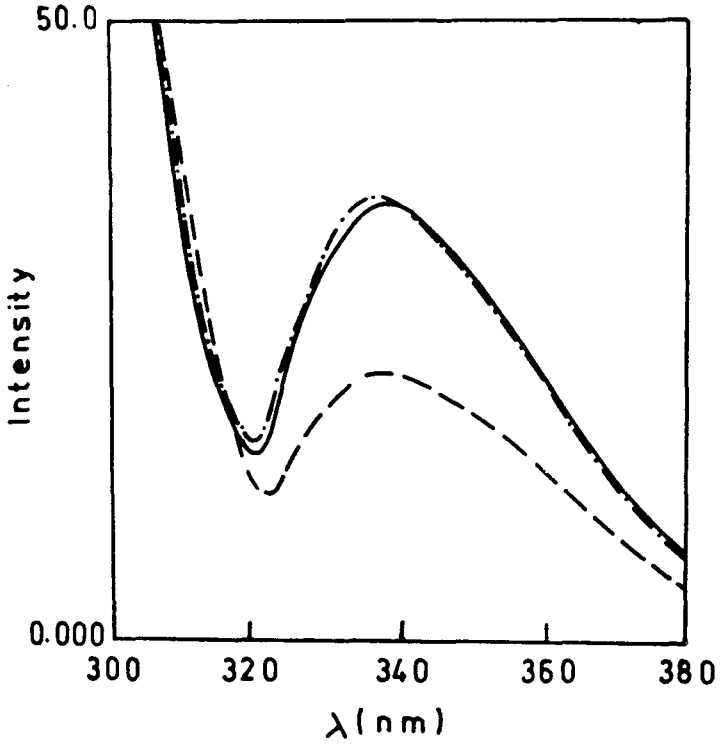

50.0
0.000
Intensity
300
320
340
360
380
λ(nm)

Figure 4.25. Fluorescence spectra of native and 30 μM zinc pretreated α_2M on reaction with methylamine and Sepharose-linked trypsin.

Fluorescence spectra of (———) α_2M alone; (— · —) α_2M reacted with methylamine; (------) α_2M reacted with Sepharose-linked trypsin; (— ·· —) 30 μM zinc reacted α_2M; (·····) 30 μM zinc reacted α_2M on methylamine treatment; (— ·· —) 30 μM zinc reacted α_2M treated with Sepharose-linked trypsin. The excitation wavelength was 280 nm, and bandwidth was 10 nm in both channels. The concentration was 0.18 μM.

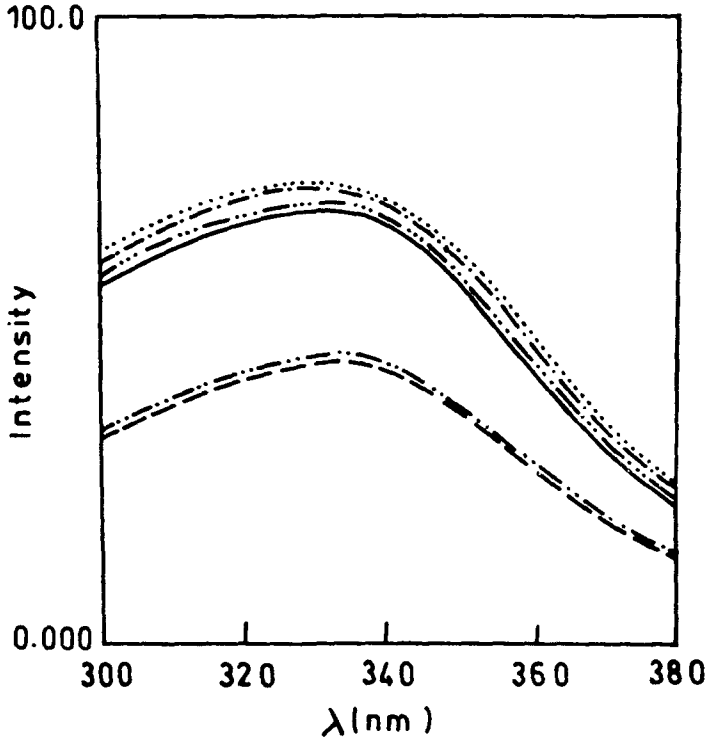

100.0
0.000
Intensity
300
320
340
360
380
λ(nm)

Figure 4.26. Fluorescence spectra of native and 200 μM treated buffalo α_2M on reaction with methylamine and Sepharose-linked trypsin.

(——) α_2M alone; (— · —) α_2M reacted with methylamine; (------) α_2M reacted with Sepharose-linked trypsin; (— ··· —) 200 μM zinc reacted α_2M; (·····) 200 μM zinc reacted α_2M on methylamine treatment; (— · · —) 200 μM zinc reacted with Sepharose-linked trypsin. The conditions of the reactions are given in methods section. The excitation wavelength was 280 nm, and bandwidth was 10 nm in both channels. The α_2M concentration was 0.18 μM.

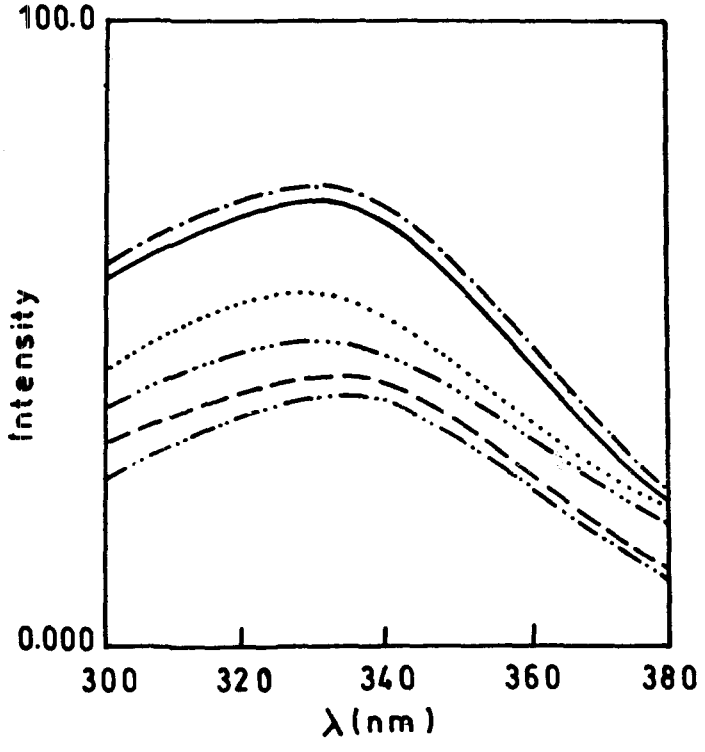
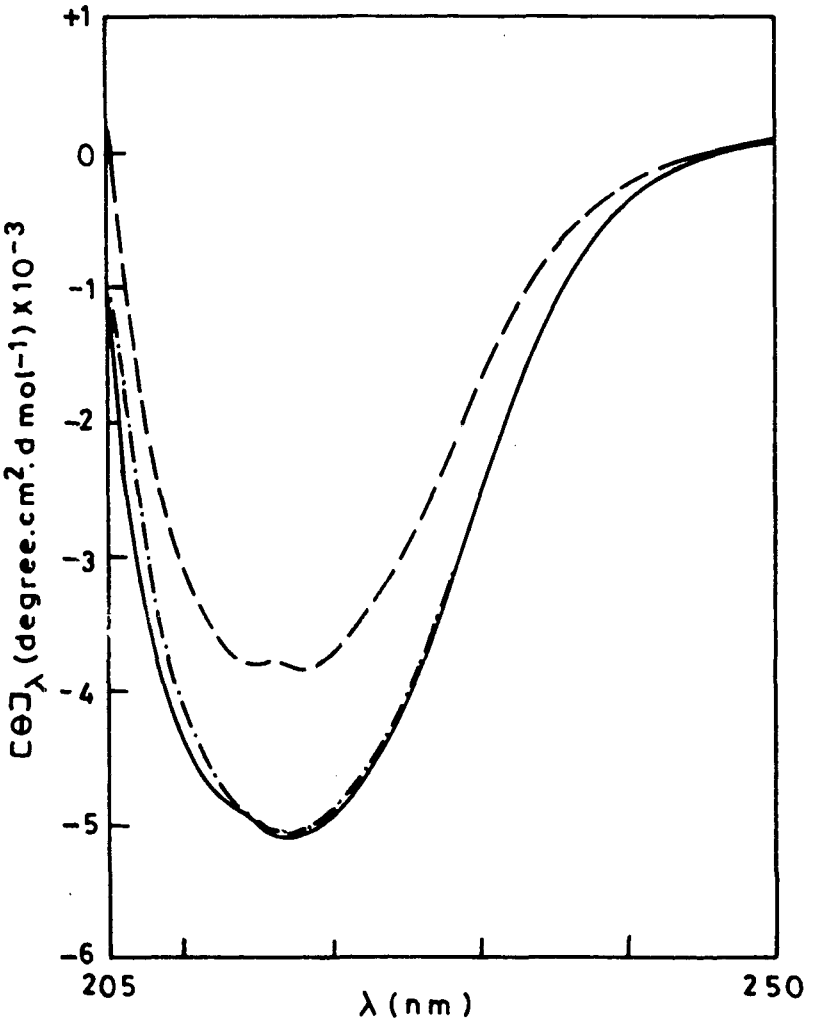

100.0
0.000
Intensity
300
320
340
360
380
λ(nm)

Figure 4.27. Far-UV circular dichroism spectra of buffalo α_2M on treatment with methylamine and immobilized trypsin.

α_2M alone and buffalo α_2M reacted with methylamine, or immobilized trypsin (——) α_2M alone; (— · —) α_2M reacted with methylamine; α_2M reacted with immobilized trypsin (-------). The conditions of the reactions with methylamine and trypsin were as given in methods. Cells with 0.1 cm path length and an α_2M concentration of 0.5 μM were used. Methylamine of the same concentrations as in the sample was included in the blank. The unit on the ordinate is mean residue ellipticity. The mean residue weight for buffalo α_2M was calculated to be 110 from amino acid analysis


+1
0
-1
-2
-3
-4
-5
-6
205
250
λ (nm)
[θ]λ (degree.cm².d mol(-1)) X 10-3

α_2M treated with 30μM zinc exhibited a CD spectrum comparable with that of native α_2M. The alteration induced by methylamine and trypsin were also comparable (Fig. 4.28). Reaction of α_2M with 200 μM zinc however, causes a marked change in the shape of the circular dichroic spectrum. While methylamine treatment caused a small increase in ellipticity, treatment with Sepharose-linked trypsin resulted a significant change in the shape of the α_2M CD spectrum (Fig. 4.29) with 25 % decrease in the mean residue ellipticity.

4.4.3.3.2. Circular dichroism in the Near-U.V. region

The circular-dichroic spectra in the near U.V region reflects the contribution of aromatic side chains, disulphide bonds, and of prosthetic groups (Kuwajima, 1989; Dryden and Weir, 1991). Figure 4.30 shows the near-UV CD spectra of buffalo α_2M in 250–300 nm range. Near-UV CD spectra of native α_2M is characterized by a band at 282 nm showing the asymmetric environment of tyrosine. Methylamine and Sepharose-linked trypsin treated buffalo α_2M showed dissimilar near UV CD spectra. A small value of MRE at 282 nm on treatment with Sepharose-linked trypsin indicates the absence of ordered structure whereas for native and methylamine treated α_2M a more ordered tertiary structure was observed (4.30).

There was no change in the CD spectral band of α_2M pre-treated with 30 μM zinc on reaction with methylamine and proteinase (Fig. 4.31). On the other hand α_2M treated with 200 μM showed subtle changes with respect to native α_2M, although the changes induced in the wavelength region around 280–290 nm were markedly different both for methylamine and Sepharose linked trypsin treated α_2M (Fig. 4.32).

4.4.4. Dissociation of α_2M by Zinc

The inhibitory activity of α_2M was determined by incubating α_2M with various

Figure 4.28. Circular dichroism spectra of 30 μM zinc treated buffalo α_2M on reaction with methylamine and Sepharose-linked trypsin in the peptide region.

(———) 30 μM zinc treated α_2M; (— · —) zinc treated α_2M reacted with methylamine; (--------) zinc treated α_2M reacted with immobilized trypsin. The condition of the reactions with methylamine and trypsin were given in methods. Cells with 0.1 cm path length and an α_2M concentration of 0.50 μM were used. Methylamine and zinc of the same concentration as in the sample were included in the blanks. The unit on ordinate is mean residue ellipticity. The mean residue weight for buffalo α_2M was calculated to be 110 from the amino acid analysis.

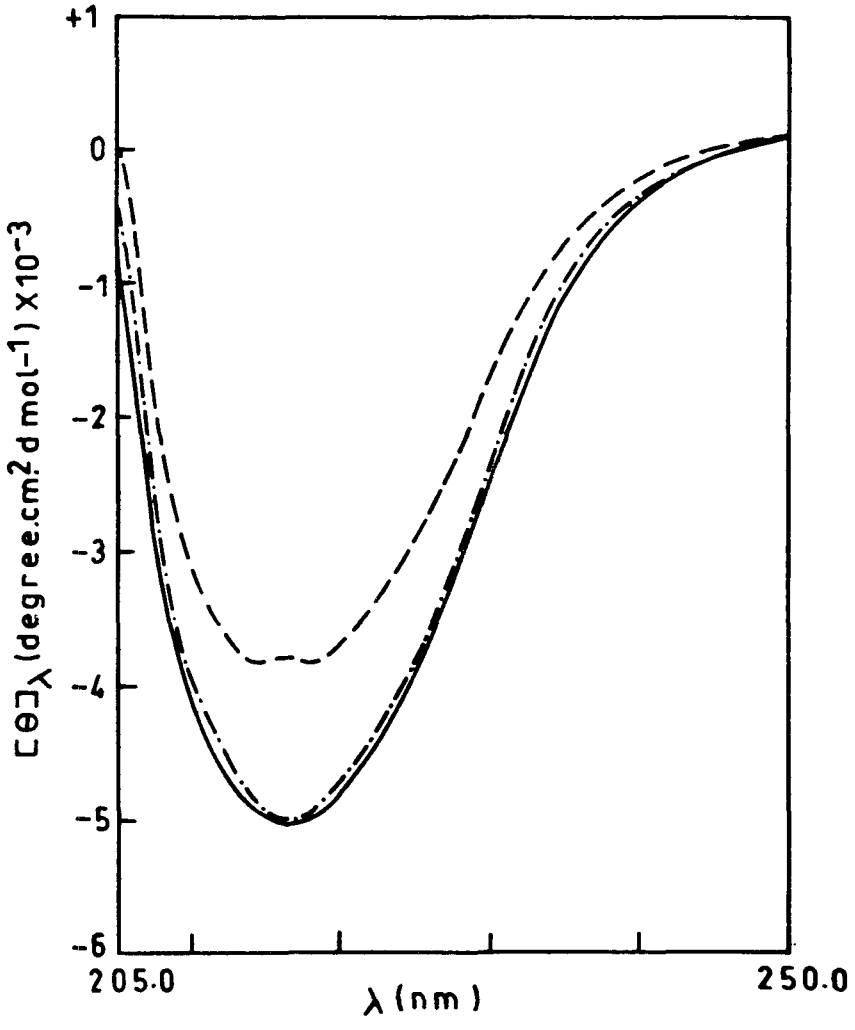

+1
0
-1
-2
-3
-4
-5
-6
$[\theta]_\lambda$ (degree.cm.^{2} d mol^{-1}) X10^{-3}
205.0
250.0
λ (nm)

Figure 4.29. Circular dichroism spectra 200 μM zinc pretreated α_2-macroglobulin and their derivatives.

CD spectra (205-250 nm) of 200 μM zinc treated α_2-macroglobulin, 0.35 mg/ml. (———) zinc reacted α_2M; (— · —) zinc bound α_2M reacted with methylamine; (---------) zinc bound α_2M treated with Sepharose linked trypsin. Cells with 0.1 cm path length were used. The Mean Residue ellipticity has been calculated with MRW of 110 calculated from amino acid analysis.

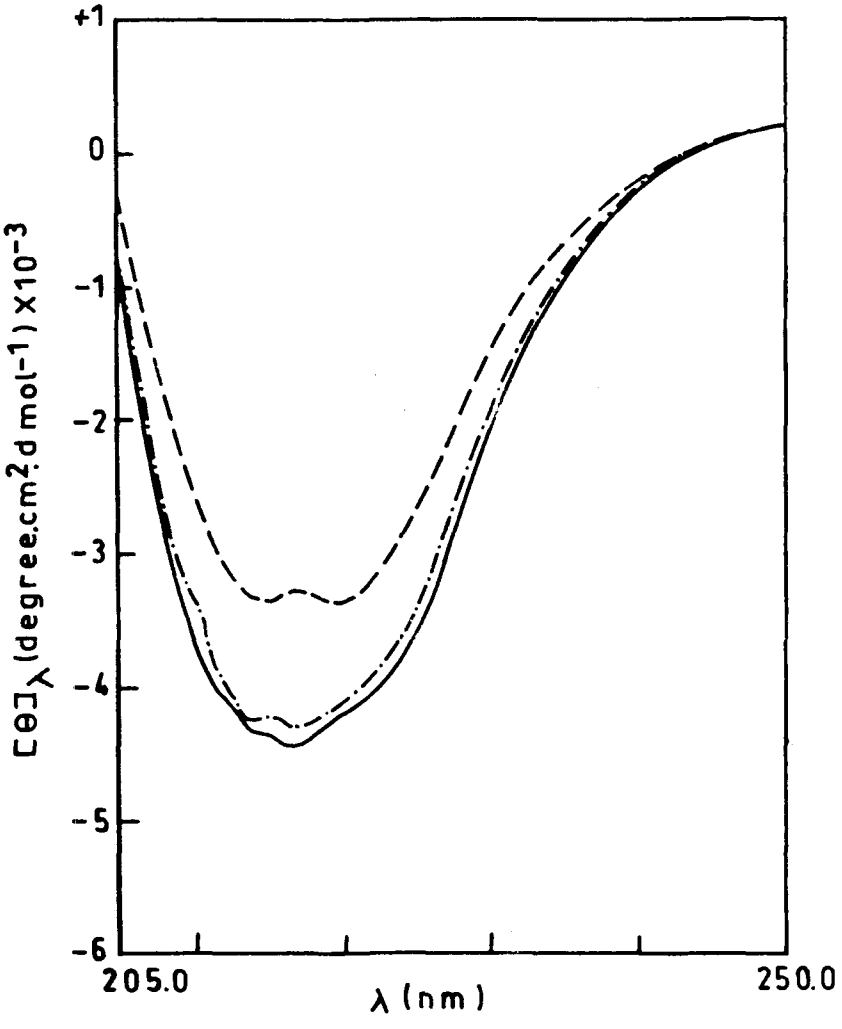

+1
0
-1
-2
-3
-4
-5
-6
205.0
250.0
λ (nm)
[θ]λ (degree.cm².d mol(-1)) X10-3

Figure 4.30. Near-U.V. circular dichroism spectra of buffalo α_2M and α_2M treated with methylamine and Sepharose-linked trypsin.

(———) α_2M alone; (— · —) α_2M allowed to react with methylamine; (--------) α_2M allowed to react with Sepharose-linked trypsin. Cells with 1 cm path length and an α_2M concentration of 1.0 μM were used. Methylamine of the same concentration as in the sample was included in the blank. The unit on the ordinate is mean residue ellipticity.

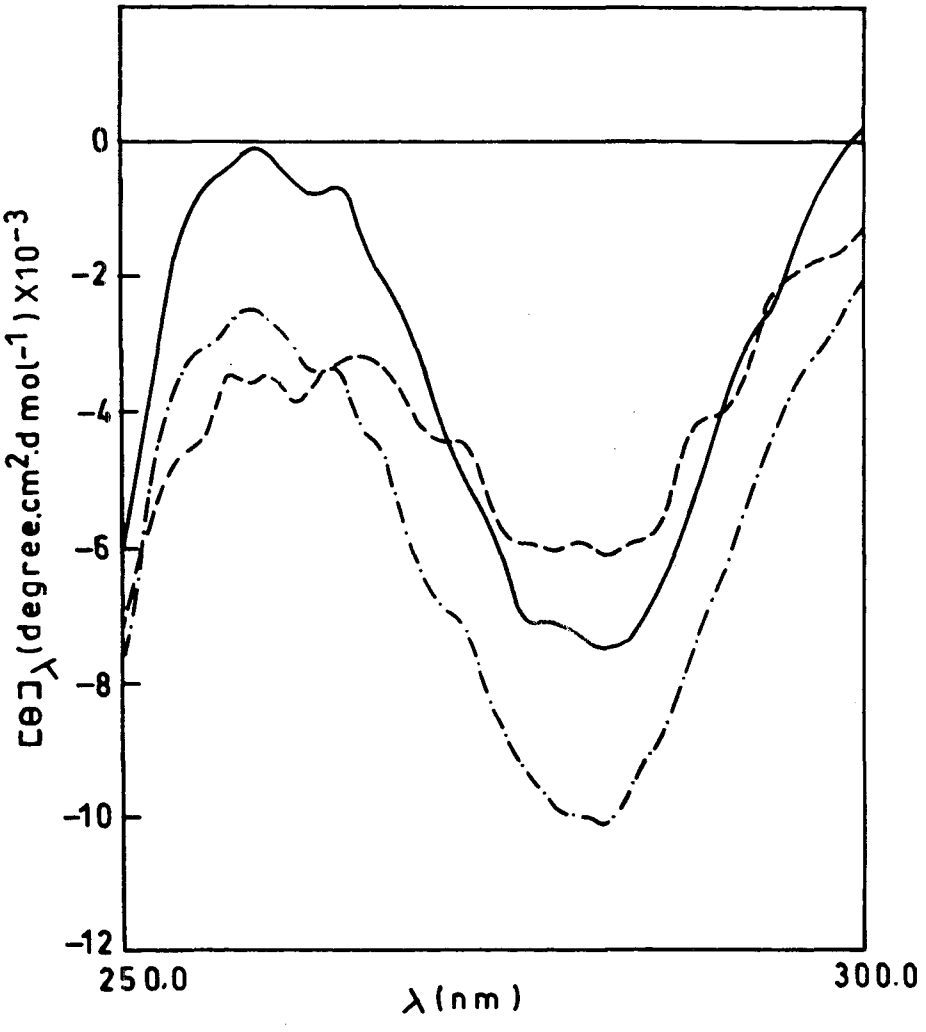

0
-2
-4
-6
-8
-10
-12
250.0
300.0
λ (nm)
[θ]λ (degree.cm2.d mol(-1)) X10-3

Figure 4.31. Circular dichroism spectra of 30 μM zinc treated α_2M on reaction with methylamine and Sepharose-linked trypsin in the aromatic region.

(——) 30 μM zinc treated α_2M; (— · —) 30 μM zinc reacted α_2M treated with methylamine; (·····) 30 μM zinc treated α_2M on reaction with Sepharose-linked trypsin. Cells with 1 cm path length and an α_2M concentration of 1.0 μM were used. The unit on the ordinate is mean residue ellipticity.

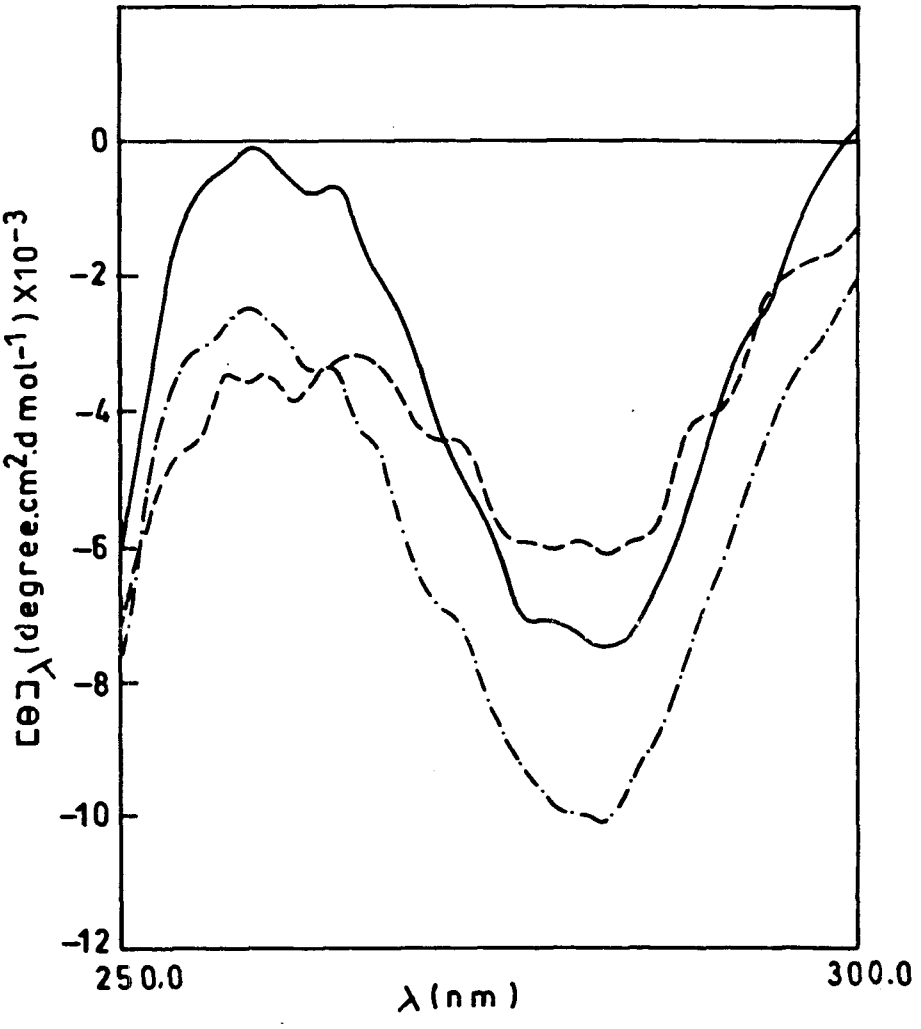
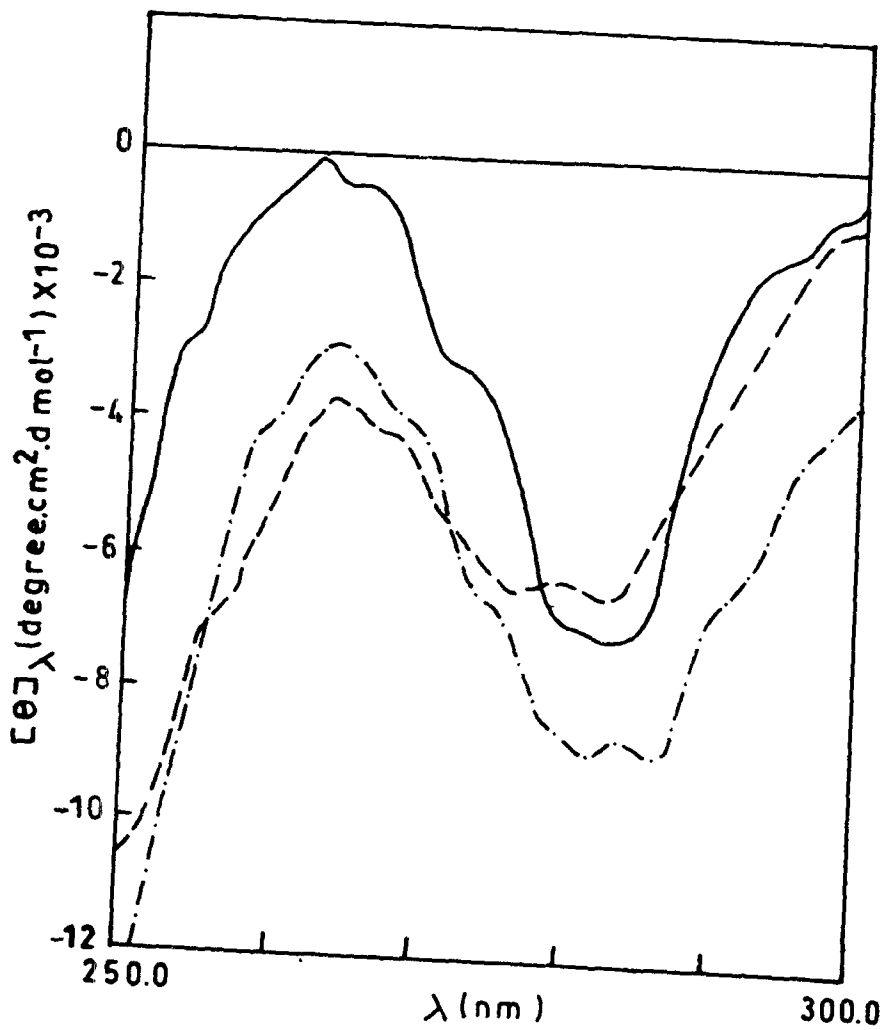

0
-2
-4
-6
-8
-10
-12
250.0
300.0
λ(nm)
[θ]λ(degree.cm2.d mol-1) X10-3

Figure 4.32. Aromatic circular dichroism spectra and 200 μM zinc treated α_2M on reaction with methylamine and Sepharose-linked trypsin.

(——) 200 μM zinc treated α_2M; (— · —) 200 μM zinc reacted α_2M followed by reaction with methylamine; (--------) 200 μM zinc pre-treated α_2M on reaction with Sepharose-linked trypsin. Cells with 1 cm path length and α_2M concentration of 1.0 μM were used. The unit on the ordinate is mean residue ellipiticity.


0
-2
-4
-6
-8
-10
-12
$[\theta]_\lambda$ (degree.cm^2.d mol^{-1}) X10^{-3}
250.0
λ (nm)
300.0

concentrations of zinc for 24 h at room temperature. With the increase in zinc concentration the inhibitory activity of α_2M decreased. Fig. 4.33 shows that at a zinc concentration of 100 μM, there is a 50% decrease in α_2M activity. Controls showed that the observed α_2M activity decrease was not related to the effects of zinc on the activity of trypsin.

The result in Fig. 4.34 demonstrates that when the native buffalo α_2M incubated for 24 h with zinc concentration up to 1mM at room temperature, the protein starts dissociating into half molecules. Complete dissociation was not observed even at 1mM zinc concentration after 48 h incubation. Molecules obtained by incubation of α_2M with 1% SDS for 90 min. at 22^0C was used as control as marker for tetrameric and dimeric forms of α_2M.

Figure 4.33. Inhibitory activity of α_2-macroglobulin in the presence of zinc.

α_2-macroglobulin samples incubated with divalent cations were assayed for activity as described under methods. α_2M activity is expressed as a percentage of the activity measured in the absence of added cation.

(●) α_2M inhibitory activity in the presence of zinc.

(○) Control.

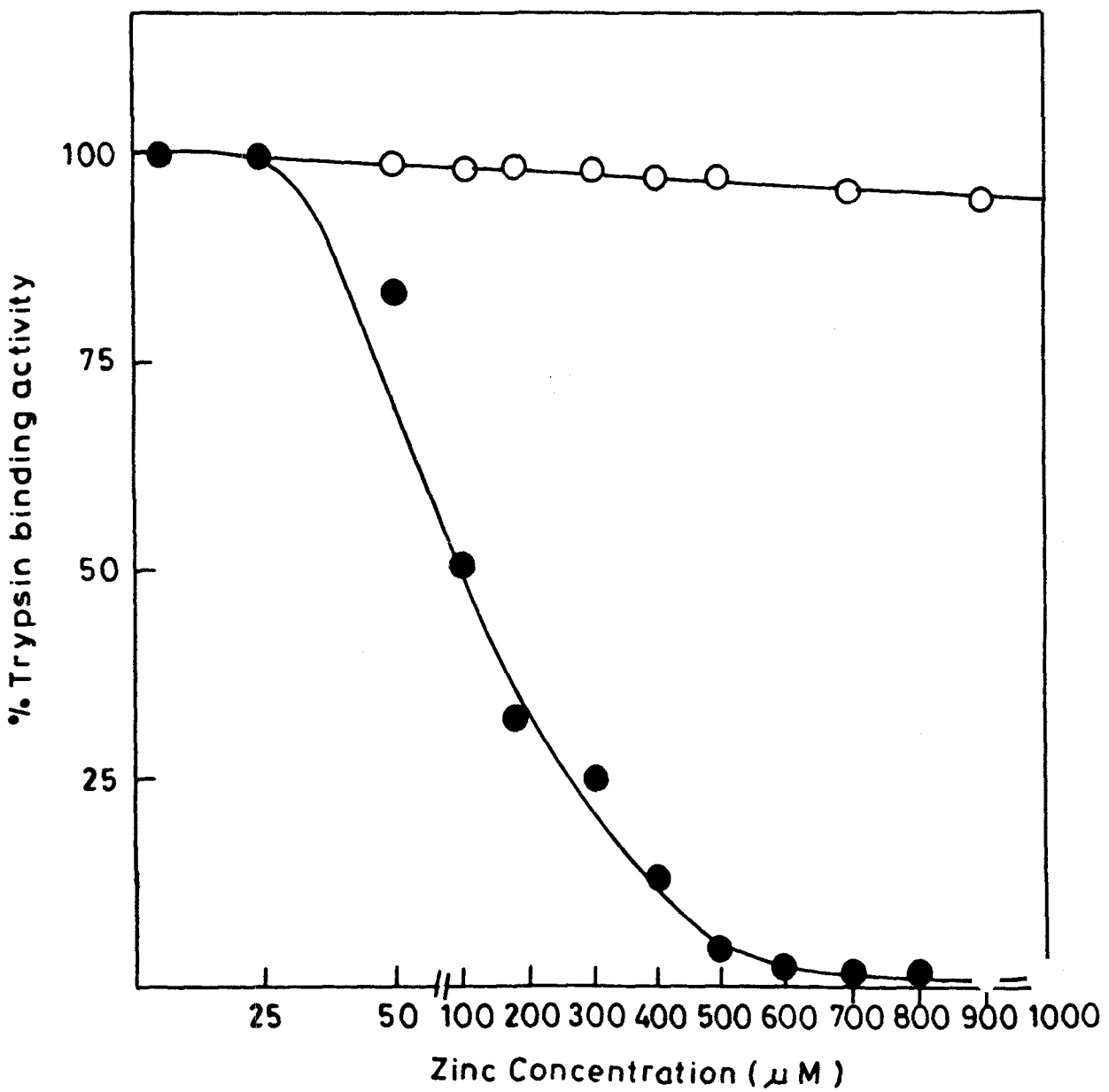
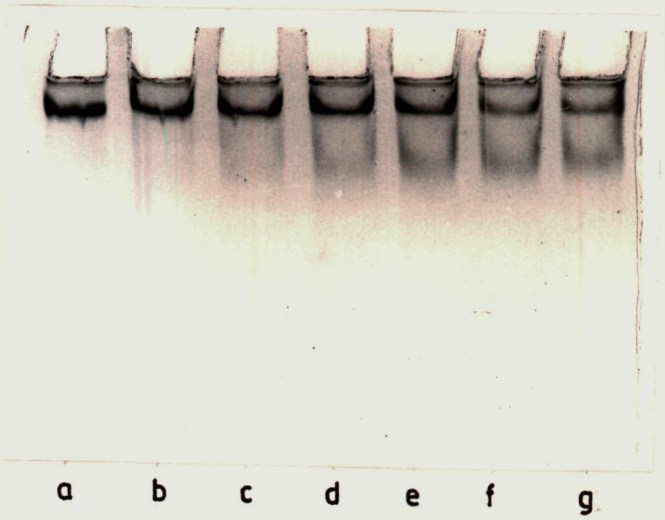

100
75
50
25
% Trypsin binding activity
25
50
100
200
300
400
500
600
700
800
900
1000
Zinc Concentration (μM)

Figure 4.34. Non-denaturing gel electrophoresis of α_2M incubated with zinc.

The effect of incubation of α_2M with different concentration of $ZnCl_2$ for 24 h at room temperature. The cation concentration are Lane a, zero; Lane b, 25 μM; Lane c, 50 μM; Lane d, 100 μM; Lane e, 500 μM; Lane f, 1000 μM. Lane g, α_2M incubated with 1% SDS for 90 min at room temperature as markers for the position of tetramer and dimer of α_2M respectively. 20 μg of protein were applied in each lane.


a
b
c
d
e
f
g

Discussion

4.5 Discussion

Alpha macroglobulins are ubiquitous proteins that occurr in diverse animal species, including the vertebrate and invertebrates (Starkey, 1982; Quigley and Armstrong, 1983a;b). The physiological roles of α-macroglobulins are indeed challenging and engrossing. The unusual mechanism of proteinase inhibition by α_2M has attracted remarkable attention and led to its purification and characterization from a variety of sources (Table 1.1). Extensive work is currently being carried out on various aspects of α_2M and several isolation procedures are available. Earlier study from this laboratory has shown that caprine α_2M is unique in several aspects and some of these characteristics were attributed to the special cleaved subunits (Khan *et al.*, 1999).

The present study involves purification and characterization of α_2M from buffalo (*Bos indicus)* blood plasma. The purification of this proteinase inhibitor was achieved using ammonium sulphate fractionation and gel filtration chromatography. A high recovery of 61% with 35-fold enrichment in specific activity is the advantage of this two step scheme of α_2M purification (Table 4.1). Homogenity of the α_2M preparation was confirmed by the presence of single band in pore-size electrophoresis (Fig. 4.2). Purification to homogeneity of human plasma α_2M normally requires additional ion-exchange step (Mirza and Saleemuddin, 1993). The ease of purification of α_2M from buffalo plasma is apparently related to the absence of large molecular weight contaminating proteins. The native inhibitor behave like a homotetramer and on treatment with SDS and β-mercaptoethanol migrated as a single band on 7.5% SDS-PAGE (Fig. 4.4). Like its human and bovine homologue (Dunn and Spiro, 1967; Nagasawa *et al.* 1970) buffalo α_2M is also glycosylated with 7.8% carbohydrate.

Various studies using SDS-gel electrophoresis, gel filtration chromatography and sedimentation equilibrium have estimated the molecular weight of the tetrameric α_2M in the range 700-800 KDa (Schultze *et al.*, 1955; Schonenberger *et al.*, 1958; Roberts, 1986;

Nagasawa *et al.*, 1970; Hudson and Koo, 1982). The molecular weight of the buffalo α_2M as determined using a Sephacryl S-300 HR column was 660 KDa (Fig. 4.6). The value is lower than that of human α_2M (Halls and Roberts, 1978) but comparable with the caprine α_2M (Khan *et al.*, 1999). Moreover, the Stokes radius of α_2M also confirms the buffalo α_2M to be more compact than that from human plasma. The value was 85^0A (Fig.4.7) as compared to the reported value of 88^0 A of the human α_2M (Roberts, 1986).

Purified buffalo α_2M when subjected to gel electrophoresis in the presence of SDS and β-mercaptoethanol yielded, a single band (Fig. 4.5). The molecular weight of the subunit of α_2M determined in this study gave a value of 165 KDa whereas, human α_2M migrates as a single 185 KDa band (Sottrup-Jensen, 1989). Inspite of its similarity in molecular dimensions the buffalo α_2M differed from its ruminant counterpart (i.e. caprine α_2M) in the nature of subunit. The caprine inhibitor is a tetramer of 146 KDa subunits which inturn are made up of two disulfide linked 110 and 36 KDa peptides (Khan *et al.*, 1999). The occurrence of cleaved subunits have also been reported in other species (Hudson *et al.*, 1987) and has been ascribed to the post translational processing of the peptide. Bovine α_2M however has an intact subunit of 185 KDa. The difference in the nature of the subunit in very closely related species may indicate lack of clear evolutionary trait but suggests abrupt specific adaptation.

Trypsinization of buffalo α_2M lead to increased mobility in pore limit and rate electrophoresis in 5% acrylamide gels (Fig. 4.8). This is similar to the characteristics "slow" to "fast" transformation of human α_2M on trypsin treatment (Barrett *et al.*, 1979; Nelles *et al.*, 1980). Sepharose-linked trypsin was capable of bringing about the transformation of α_2M to the "fast" form albeit at a slower rate (Fig. 4.9) indicating that immobilization process does not abolish the bait region cleaving ability of the proteinase. It has been shown that treatment of human α_2M with solid support attached trypsin

results in the formation of empty traps that resemble the trypsin treated forms in several respects (Mirza and Saleemuddin, 1993). Similarity in the nature of α_2M transformation induced by soluble and immobilized chymotrypsin has been demonstrated by image processing of the electron microscopy (Biosset *et al.*, 1991a;b).

αMs from a variety of sources function as "molecular traps" for broad spectrum proteinases (Barrett and Starkey, 1973) rather than as inhibitors (Sottrup-Jensen, 1987; Sottrup-Jensen, 1989; Roberts, 1986). Buffalo α_2M appears to be no exception and exhibits inhibitory activity and protection of amidolytic activity to trypsin (pH 8.0), chymotrypsin (pH 8.0), pronase E (pH 8.0), proteinase K (pH 8.0) and bromelain (pH 6.8) as shown in Table 4.3. This is apparently related to the presence of susceptible bonds in the bait region of the molecule.

It is evident from several studies that the reaction of α_2M with methylamine is a complex phenomenon involving random attack on the thiolesters (Feinman, 1983; Sottrup-Jensen, 1987; Strickland and Bhattacharya, 1984; Larson *et al.*, 1985). Incubation of buffalo α_2M with methylamine, resulted only in a very small increase in electrophoretic mobility (Fig. 4.10). Nearly half the original activity of buffalo α_2M is retained by the α_2M after treatment with methylamine (Table 4.2). This suggests that the thiol esters of buffalo α_2M are more stable to nucleophilic attack by methylamine than those of human α_2M (Gonias *et al.*, 1982). Somewhat similar behaviour is shown by bovine and caprine inhibitor (Nagasawa *et al.*, 1970; Khan *et al.*, 1999). It is interesting to note that the methylamine treated human α_2M is completely devoid of the ability to inhibit or entrap proteinase (Gonias *et al.*, 1982), while the inhibitory activity of equine α_2M remains completely unaffected by methylamine (Motoshima *et al.*, 1988).

The number of methylamine or trypsin sensitive thiol esters present in buffalo α_2M was determined by the quantitation of the number of excess free thiols generated as a result of these treatments. Methylamine liberated only 2 moles of thiols per mole of

α_2M while those liberated in response to trypsin treatment were 4 (Table 4.4). Although, buffalo α_2M like its human and bovine counter part has 4 moles of thiol ester per mole but the accessibility of two among these seems to be restricted even to low molecular weight molecule i.e. methylamine. Although the physiological significance of the presence of thiol esters of unequal reactivity is difficult to predict, their occurance has also been reported in caprine α_2M (Khan *et al.*, 1999). Trypsinization of methylamine pretreated α_2M caused further release of two thiol esters and converted the intermediate mobility species to "fast" from on 5% gel (Fig. 4.10).

In human α_2M the structural constraints released by the thiol ester cleavage are transmitted to the other parts of the molecule, ultimately causing perturbation in the thiol ester domain of the adjacent subunit and the increase in the accessibility of thiol esters to methylamine (Gonias *et al.*, 1982 Dangott *et al.*, 1983). Apparently these exert an opposite effect as in buffalo α_2M and conformational changes occuring as a result of cleavage of two thiol esters restrict the accessibility of the remaining two esters. Alternatively, the recalcitrance of two thiolesters of buffalo α_2M may result from their location in deep hydrophobic cleft in manner that makes them inaccessible even to small molecular weight amine.

The stoichiometry of inhibition of trypsin showed that 1.0 mole of intact buffalo α_2M entrapped a maximum of 1.0 mole of enzyme as shown in Fig. 4.11 which is lower than that of human inhibitor. This behaviour may be related to the structural features of buffalo α_2M that promote a higher extent of non-productive activation by cleavage of the bait region before binding of proteinase (Ganrot, 1966) or due to smaller dimensions of the buffalo inhibitor (Fig. 4.7).

The amino acid composition of buffalo α_2M bore remarkable similarities with those of human, goat and mouse α_2Ms (Dunn and Spiro, 1967; Hudson *et al.*, 1987; Khan *et al.*, 1999). The concentrations of Asp, Thr and Pro were lower significantly while those

of Gly and Ala were higher (Table 4.5). The similarities are also reflected in the cross reactivity of the anti-buffalo α_2M with goat and human α_2M (Fig 4.13).

The dimer-dimer interactions in the tetrameric α_2Ms play an important role in the trapping of proteinase and regulation of conformational changes that accompany proteolytic attack (Jensen, 1993). The tetramerization of α_2M may have functional implications on the biological functions of the inhibitor, which may involve the simultaneously evolved contact zone. The significance of the contact zone has been emphasized for human α_2M (Shanbhag *et al.*, 1997). Curiosity to investigate the role of contact zone as a functional site in non-human tetrameric α_2M's led us to carry out the studies on the dissociation of the buffalo inhibitor.

Dissociation of α_2M along non-covalent axis by urea, guanidine hydrochloride, SDS, metal ions of +2 oxidation State (Zn, Cd, Hg, Cu, Ni) and chaotropic anion has been shown to generate half molecules (Sottrup-Jensen *et al.*, 1980; Pochon *et al.*, 1987; Pratt and Pizzo, 1984; Roche *et al.*, 1988; Sjoberg *et al.*, 1991; Shanbhag *et al.*, 1996). The chaotropic anion thiocyanate has been used to dissociate buffalo α_2M and α_2M-$MeNH_2$ complex through non-covalent axis in this study. The result in Fig. 4.14 clearly shows that thiocyanate dissociates α_2M into half molecules with an electrophoretic mobility comparable to that obtained by incubating the protein with 1% SDS (Harpel *et al.*, 1979). α_2M-$MeNH_2$ dissociates at far higher concentration of thiocyanate than that required for native α_2M (Fig. 4.15).

It is known that the chaotropic thiocyanate anion modifies the structure of water thereby disrupts the hydrophobic interactions (Hamaguchi and Geiduschek, 1962). Dissociation of the tetramer in presence of NaSCN suggests the involvement of hydrophobic amino acids in the association between the non-covalent bonded subunits in α_2M. The observed difference in the dissociation profile between native and methylamine reactive α_2M is indicative of the increased interaction between the contact zone which

inturn are also controlled by the conformational changes induced in the protein on the thiol ester cleavage or by cleavage of both thiol ester and bait region. Human α_2M-$MeNH_2$ also dissociates to a lower extent than the native α_2M in presence of NaSCN (Shanbhag *et al.*, 1996).

The Stokes radius of the buffalo α_2M half molecule generated by sodium thiocyanate was similar to that obtained by dissociation with 4M urea (Table 4.6). Neither urea nor NaSCN cleave disulphide bonds (Liu *et al.*, 1987; Shanbhag *et al.*, 1996). Thus, the α_2M half molecule generated in this study is the likely result of the disruption of only the non-covalent interactions. These observations taken together indicate that no major conformational change may have occurred during dissociation of tetrameric α_2M into half molecules under the conditions used and the dimers may remain in an undenatured state. In support of this the observed stokes radius for the fraction of α_2M that remained undissociated on treatment with 1.6M NaSCN was comparable with that of native α_2M (Table 4.6).

Fig. 4.16 and 4.17 shows the difference in the intrinsic fluorescence of native α_2M and that treated with 1.6M NaSCN. The dissociation of tetramer into half molecule causes a decrease in the intrinsic fluorescence with a slight red shift of the emission maximum, after 80 min. incubation with the anionic salt. This decrease in fluorescence suggest that some conformational changes occur as a consequence of dissociation in which the environment of the hydrophobic amino acid is altered. The dissociation of human α_2M into half molecules induced by NaSCN results in conformational changes resembling those that occur to thiol ester cleavage in the inhibitor and treatment of the resulting half molecules with methylamine caused no significant additional conformational alteration (Shanbhag *et al.*, 1997). The results shows the presence of aromatic amino acid residues at the contact zone and that these fluorophores are gradually brought into a polar environment. The fluorescence behaviour of the exposed aromatic amino acid of a protein may also be influenced by the composition of the

solvent even in the absence of any significant conformational changes and such effect have been used to probe the solvent accessibility of aromatic residues of a protein (Creighton, 1991.). Addition of sodium thiocyanate is known to cause an increase in the polarity of the solvent (increased refractive index) and hence may cause a decrease in fluorescence intensity and shift in emission maximum (Fig. 4.16 and 4.17). It is also possible that the tyrosyl hydroxyl groups of proteins are involved in hydrogen bonding with the solvent molecules (Creighton, 1991).

The 90% change in the fluorescence (ΔF max) resulting from incubation with NaSCN for native α_2M both at (λ ex) 280 and 295 nm was reached far more rapidly than that of α_2M-$MeNH_2$ (Fig. 4.18 and 4.19). That attainment of ΔF max necessitates approximately twice the time for α_2M-$MeNH_2$ complex than for native α_2M. This is a further indication of the enhanced interactions between the non-covalently associated subunits in the methylamine treated α_2M. The methylamine induced enhancement in interaction between the dimers may be far more stronger in case of human α_2M that requires a six-fold increased concentration of thiocyanate to induce a similar rates of changes in fluorescence comparable to the native α_2M (Shanbhag *et al.*, 1997). The difference is evidently related to the fact that only two thiol esters are cleaved on treatment of the buffalo α_2M with methylamine while in case of human α_2M all the four thiol esters are equally reactive to the methylamine treatment. It is also likely that the areas of contact between the disulphide bonded dimers are smaller due to smaller dimensions of the buffalo α_2M. It is however not clear if the two thiol esters cleaved in buffalo α_2M are those in disulphide linked or non-covalently associated subunits.

α_2M is also a major zinc binding protein of human plasma (Parisi and Vallee, 1970; Adham *et al.*, 1977). Equilibrium dialysis of the binding of ^{65}Zn by α_2M at pH 7.9 showed heterogenous binding which could be attributed to two classes of binding sites (Pratt and Pizzo, 1984). According to the Delain model (1988) the contact zone between two dimers may contain two strong Zn^{2+} binding sites (Gettins and Cunningham, 1986).

The physiological zinc concentration in buffalo plasma as estimated by the method of Song *et al.* (1976) varies in the range of 18-20 μM. About 400-625 μg Zn/g of purified protein was quantitiated in buffalo while about 320-770μg of zinc has been reported per g of human α_2M (Parisi and Vallee, 1970).

The effect of zinc on the amidolytic activity of trypsin shown in Fig. 4.20 indicates that trypsin binding activity does not depend on the presence of zinc in this protein, since removal of nearly all the zinc by EDTA results in no loss of trypsin binding activity. Increase in zinc concentration beyond 30 μM results in marked loss of enzyme binding activity. Removal of unbound zinc from α_2M against buffer containing EDTA did not restore its trypsin binding activity (Fig 4.20).

The buffalo α_2M exposed to either low or high zinc concentration responded to methylamine and Sepharose linked trypsin treatment as the native α_2M (Fig 4.21) by undergoing the characterstic "slow" to "fast" transformation.

Surprisingly, the α_2M treated with 200 μM zinc lacked the ability to protect trypsin against inhibition by SBTI (Fig. 4.20). Considering that bait region cleavage and accompanying gross conformational changes occur in the preparation treated with trypsin (Fig 4.20). The behaviour may be related to very rapid closure of the "Trap" after bait region cleavage without entrapment. Alternatively, small alteration in the interactions between the covalently associated dimers may restrict the steric protection to the inhibitor associated trypsin from reaction with SBTI.

The intrinsic fluorescence spectra were clearly different for methylamine and proteinase treated buffalo α_2M (Fig. 4.23). The emission maximum for native α_2M is 333 nm (at λ ex 280 nm). Treatment of native α_2M with Sepharose-linked trypsin caused a drastic decrease in fluorescence intensity with a small red shift. Exposure of aromatic amino acids to a polar environment results in a decrease in fluorescense intensity

(Friedfelder, 1984). Methylamine causes 3nm blue shift with slight enhancement in fluorescence suggesting of burial of aromatic residues inside the protein. These findings clearly suggests that the conformational alteration accompanying trypsin and methylamine treatments are remarkably different in buffalo α_2M. The emission maximum for native α_2M at (λ ex) 295 nm was observed at 339 nm (Fig. 4.24) which is characteristic for tryptophanyl fluorescence. Both methylamine and immobilized trypsin treated α_2M showed 3 nm blue shift in the emission maximum (Fig. 4.24). Interestingly results from Fig. 4.23 and 4.24 indicate that while tryptophans may get buried in the hydrophobic environment whereas tyrosine are exposed to the solvent after trypsin or methylamine treatment. Trypsin treatment induced gross conformational changes in buffalo α_2M as has been observed in case of human, bovine and equine α_2M using fluorescence measurements (Bjork and Fish, 1982; Bjork *et al.*, 1985; Motoshima *et al.*, 1988). The buffalo inhibitor however appears unique in that it exhibits decrease in fluorescence on trypsinization but other αMs showed enhancement. Use of Sepharose-linked trypsin in the experiments eliminates the interference by the proteinase as this does not results in the entrapment of the former and leads to the formation of "empty closed trap" (Mirza and Saleemuddin, 1993). The principal difference between this study and those of Bjork *et al.* (1982; 1985) and Motoshima *et al.* (1988) is the use of soluble trypsin by the latter for inducing the conformational alterations. It is now well known that the entrapment of the challenging soluble proteinase in α_2M results the observed alterations in fluorescence that may actually represent a combined contribution of the accompanying conformational alteration and those of the entrapped proteinase. The effect of methylamine on buffalo α_2M was however marginal suggesting minimal conformational alterations as observed in case of the bovine and equine α_2Ms (Bjork *et al.*, 1985; Motoshima *et al.*, 1988).

Exposure to zinc yielded interesting alteration in buffalo α_2M in response to the methylamine and trypsin. Pretreatment of the inhibitor with 30 μM zinc resulted in no major alteration in the fluorescence behaviour (Fig. 4.25), while exposure to 200 μM zinc

caused a 30% decrease in fluorescence intensity (Fig. 4.26). It may recalled that buffalo α_2M exposed to 200 μM zinc loses the ability to protect trypsin from SBTI inhibition although it undergoes the characteristic proteolysis and methylamine induced alteration (Fig. 4.20).

The observed decrease in fluorescence intensity in reaction of α_2M with Sepharose-linked trypsin may be related to movement of tryptophan and tyrosine in the proximity of ionized amino acids thereby resulting in the quenching of the fluorescence. However, it is also possible that tryptophans shift in a region of a protein where movement of the indole ring is hindered (Friedfelder, 1984).

Significant change in shape and magnitude in circular dichroic spectra of α_2M treated with methylamine and trypsin has been reported for human α_2M (Bjork and Fish, 1982). For buffalo α_2M, minimal alteration was observed with methylamine similar to bovine and equine α_2M (Bjork *et al.*, 1985; Motoshima *et al.*, 1988) whereas Sepharose-linked trypsin caused a sharp decrease in ellipticity with a change in the shape of the spectra (Fig. 4.27).

The major difference between human and buffalo α_2Ms lies in the behaviour after treatment with methylamine. Following the cleavage of thiol ester bond by methylamine, human α_2M undergoes extensive conformational change that alters both the secondary and tertiary structure as well as the shape of the protein (Bjork and Fish, 1982, Gonias *et al.*, 1982; Dangott *et al.*, 1983).

The observed changes in the buffalo α_2M circular dichroic spectra resulting from reaction with methylamine and Sepharose-linked trypsin was analyzed by the method of Chen *et al.* (1972). Reaction with trypsin decreased α_2M α-helix content from approximately 9 to 4% whereas methylamine caused no change. Gonias *et al.* (1982) reported a decrease in α-helix of about 9-10% from 14% for human α_2M . For all three

forms of α_2M (native, methylamine and trypsin treated) the majority of the remaining may be β-ordered and unordered structure (Bjork and Fish, 1982). The circular dichoric spectrum of the 30μM zinc treated buffalo α_2M on reaction with both methylamine and Sepharose-linked trypsin showed similar pattern as native α_2M (Fig. 4.29).

There was a loss of about 2% secondary structure of 200μM zinc pretreated α_2M. Methylamine treatment of zinc treated form resulted only marginal change in the spectra as compared to trypsin treatment that caused a very significant decrease in mean residue ellipticity as shown in Fig. 4.30.

The near-U.V. circular dichroism spectral changes as displayed in Fig. 4.31 may not be the true representation of major conformational changes in the protein. The spectra indicate alterations in the immediate structural and electronic environment of the aromatic amino acid residue resulting from interaction with methylamine or proteinase with the inhibitor (Creighton, 1991). The mean residue rotation of α_2M treated with Sepharose-linked trypsin is decreased indicating the presence of less ordered structure as compared to native and methylamine treated α_2M (Fig. 4.31).

In conclusion it has been shown that buffalo α_2M inspite of its smaller dimensions exhibits majority of the characteristics features. Investigations of the zinc treated buffalo α_2M suggests that in presence of high concentration of the cation the inhibitor retains several characteristics features but irreversibly looses the ability to entrap and protect the protease from large molecular weight inhibitors.

Summary

5.0 SUMMARY

Buffalo plasma α_2M was purified to an apparent homogeniety by ammonium sulphate fractionation and gel filtration chromatography. The protein was purified 35- fold with an yield of about 61%. The molecular weight determined by gel filtration and SDS-PAGE was 660 KDa. SDS-PAGE in presence of thiol reductant dissociated the protein into quarter subunits with a molecular weight of 165 KDa. The stokes radius of buffalo α_2M calculated from gel filtration data was 85^0 A.

The purified buffalo α_2M migrated as a single band on polyacrylamide gel electrophoresis and showed an increased mobility after reaction with trypsin. Methylamine caused only a small change in electrophoretic mobility. Trypsinization of the methylamine treated preparation completed the transformation to the fast form.

Studies of methylamine revealed that buffalo α_2M has thiol esters of unequal reactivity. Two of the four thiol esters appeared recalcitrant to methylamine treatment. The carbohydrate composition of the purified protein was 7.8% dry weight of the molecule. The amino acid composition of buffalo α_2M appeared typical of α_2Ms except for the deficient in proline and aspartic acid and higher content of alanine. Buffalo α_2M exhibited good immunological cross reactivity against human and goat α_2M.

Sodium thiocyanate at 1.2M or higher concentration dissociated the native buffalo α_2M into half molecules consisting of two disulphide bonded subunits. Methylamine treatment rendered the molecule more resistant to dissociation than native α_2M. The observed flourescence change indicates that conformational alteration occurs gradually on exposure to sodium thiocyanate.

The physiological zinc concentration of buffalo plasma was about 18-20 μM. α_2M pre-treated with upto 30μM zinc retained most properties of native α_2M, while α_2M treated with 200μM zinc exhibited an irreversible loss in activity, although it displayed the characterstic proteolysis and methylamine induced alterations in electrophoretic mobility.

Trypsin treatment resulted in a significant decrease in intrinsic fluorescence of buffalo α_2M whereas methylamine caused only marginal alterations. The magnitude of conformational changes occurring on methylamine and trypsin treatment were markedly higher in case of the α_2M pretreated with 200 μM zinc. The changes in the CD spectrum of buffalo α_2M were also very small on methylamine treatment whereas loss in ellipticity was remarkable on treatment with Sepharose-linked trypsin. Treatment of buffalo α_2M with 200 μM zinc resulted in significant alteration in the CD spectrum also after treatment with methylamine or trypsin. Prolonged incubation with high concentration of the metal ion caused the dissociation of α_2M into half molecules.

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6.0 BIBLIOGRAPHY

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